Induction of bovine respiratory syncytial virus vaccinal immunity

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Induction of bovine respiratory syncytial virus vaccinal immunity

by

Muhammad Zulfiqar

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

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In Charge of Major Work

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For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1995
DEDICATION

I would like to dedicate this humble accomplishment of mine to those individuals who made more sacrifices than I did in order for this work to be completed: my parents, my daughters Ammara and Saadia, who stayed back home with their grandmother for three long years, my son Shaheer, my daughter Ausma, my wife Shahnaz and my parents-in-law. Without their untiring support and continuous love, I would not have been able to complete this work.
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GENERAL INTRODUCTION

In spite of all efforts and modern scientific developments, bovine respiratory disease (BRD) continues to be an enigma for the cattle industry of many countries including the United States of America. It has been estimated that 40-80% of all diseases of cattle involve the respiratory system (Lillie, 1974). In the United States alone it causes a loss of 250-750 million dollars annually (Loan, 1983). Baker (1984) cited a 1977 Livestock Conservation Institute estimate of a one billion dollar loss due to BRD per year in North America.

Bovine respiratory syncytial virus (BRSV) is one of the most frequently occurring and virulent etiological agents of respiratory tract disease in calves. It is considered to be a major contributor to the incidence of respiratory tract disease in nursing beef, feed lot and dairy calves (Duncan and Potgieter, 1993; Kimman et al., 1988; Baker and Frey, 1985). The virus causes an acute pneumonia with high morbidity and appreciable mortality in some herds. There are also indications that BRSV infection can predispose to bacterial pneumonia resulting in increased severity of the disease (Al-Darraji et al., 1982). Seroepizootiological surveys and virus isolation have indicated that exposure of cattle to BRSV is widespread in many countries (Pernthaner et al., 1990; Burgu et al., 1990; Kimman et al., 1988; Baker et al., 1986; Mahin et al., 1985; Elazhary et al., 1980).
Bovine respiratory syncytial virus is an enveloped, single stranded, negative sense RNA virus that has been classified with human respiratory syncytial virus (HRSV) as Pneumovirus in the family Paramyxoviridae (Kingsbury et al., 1978). The viruses are antigenically related (Lerch et al., 1989) and cause the greatest morbidity in their respective species during the first six months of life when maternal immunity is still present. Reinfections are common and difficulties have been encountered in providing protection through live or inactivated vaccines against respiratory syncytial viruses (RSV) (Stott and Taylor, 1985). A major limitation of these vaccines is that maternally derived immunity to BRSV suppresses an active immune response to vaccination in calves (Kimman et al., 1987; Westenbrink et al., 1989). The basis for this suppression is not well understood. It may result from the blocking effect of the specific anti-viral antibodies. The phenomenon of antibody-mediated immunosuppression has been demonstrated in cotton rats in which the passive transfer of hyperimmune RSV antiserum suppressed the immune response to RSV glycoproteins but not to vaccinia virus antigen (Murphy et al., 1988).

The genome of the respiratory syncytial virus codes ten proteins, including two nonstructural (1B and 1C) and eight structural proteins (Mallipeddi et al., 1990; McIntosh and Chanock, 1990). The major glycoprotein (G), fusion protein (F), 1A protein and 22Kd proteins are components of the viral envelope. The G protein mediates attachment of the virion to the cell membrane. The F protein mediates fusion between the cell membrane and the viral envelope. The nucleocapsid contains
the nucleocapsid protein (N), the phosphoprotein (P), and the large protein (L). The matrix protein (M) forms a structural layer between the envelope and the nucleocapsid (McIntosh and Chanock et al., 1990). Antibodies to all the structural proteins develop in convalescent calves (6 to 7 months old), but these are predominantly directed against the F and N proteins (Westenbrink et al., 1989). Similarly, in a limited study, 2 to 3 week old calves have been reported to have moderate levels of maternal antibodies to the F and N proteins against BRSV (Westenbrink et al., 1989). However no definite studies appear to have been published regarding the specificity and mean duration of maternal antibodies in calves.

A number of workers have analyzed the antibody response to N protein of HRSV in experimental animals with variable results. King et al. (1987) reported that N protein expressed in vaccinia virus afforded a significant degree of protection in BALB/c mice. Although vaccination with the recombinant expressing N protein resulted in a significant reduction in titer in the lung of the mice following live virus challenge, the protection was not as solid as provided by G and F proteins expressed and used similarly (Stott et al., 1986; Wertz et al., 1987). Peretz et al. (1992) have reported that G and F, but not N protein induces protection in BALB/c mice. Immunity induced by G or F proteins lasts longer than that induced by N protein (Connors et al., 1991). The N protein probably plays a role in RSV-specific cytolytic T-cell responses (McIntosh and Chanock, 1990) because N protein served as
a target for RSV-specific Tc cells in both mouse and human studies (Bangham et al., 1986; King et al., 1987). It is therefore reasonable to assume that the N protein of BRSV may also serve as a target for bovine Tc cells. These observations suggest that N protein is not as immunogenic as the G and F proteins and thus maternal antibody to the N protein may drop below detectable level sooner than the antibody to the F protein. Our own preliminary studies (unpublished data) in which the specificity and duration of maternal antibody was studied suggested that antibody to the N protein drops below detectable levels before antibody to the F protein. Consequently, it may be possible to immunize calves with the N protein in the presence of levels of maternal antibody that interfere with responses to other viral proteins. The role of nucleocapsid protein as a protective antigen has also been described for the morphologically similar influenza virus (Wraith et al., 1987).

As far as ascertainable from the literature the N protein of BRSV has not been used as immunogen in any form. Therefore the purpose of this study was to:

1) study the morphogenesis of BRSV strain 375 grown in Vero cells,

2) generate monoclonal antibodies (MAbs) against the N protein of BRSV and to characterize these by ELISA, indirect immunofluorescence, epitope mapping, and radioimmunoprecipitation assay, and

3) use solid matrix antibody-antigen complexes containing N protein of BRSV to investigate the immunogenicity of the N protein and to determine its carrier effect on the subsequent vaccination (challenge) with heat inactivated virus.
Dissertation Organization

This dissertation consists of a general introduction, a review of literature, three manuscripts, a general summary, and literature cited. The first manuscript was written for submission to Archives of Virology. The second and third manuscripts were written for submission to the American Journal of Veterinary Research. The format used for literature cited in the manuscripts is according to the requirement of each journal. Literature cited within the general introduction, literature review, and general summary is presented at the end of the dissertation.
LITERATURE REVIEW

Historical Background and Prevalence of the Virus

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. Since it caused clinical signs like coryza they named this virus chimpanzee coryza agent (CAA). The epizootic was not severe, involving some 20 animals; however, further studies with the virus have been very extensive, opening broad areas in human as well as bovine respiratory disease research. Experimental infection in a number of laboratory animals including day-old mice, weanling hamsters, young rabbits, guinea pigs, etc. indicated that only chimpanzees were affected by this virus. A serological survey indicated that the CAA was not antigenically related to many other viruses being studied at that time. In the course of this study, a laboratory worker developed a moderate respiratory infection. Although CAA could not be isolated from him, there was serological evidence that this virus was involved in the illness. This finding led to a serological survey which indicated a broad antibody response in the human population, which in turn stimulated the isolation of CAA or a similar agent from humans with respiratory disease.

The following year antigenically identical viruses were isolated (Chanock et al., 1957) from two infants with lower respiratory tract disease. These isolates were named the Snyder and Long strains after the patients from whom they were isolated. The cytopathic effects (CPE) of these isolates were indistinguishable from those
produced by CAA. A close antigenic relationship was found between CAA and the new isolates by complement fixation and cross-neutralization tests. Since these agents and CAA appeared to be antigenically identical, and because of the characteristic syncytium production in cell culture, the isolates were grouped together as respiratory syncytial viruses (Chanock et al., 1957).

Like human respiratory syncytial virus (HRSV), antibodies to bovine respiratory syncytial virus (BRSV) were also reported before isolation of the virus. In 1968 English investigators working on HRSV realized that the calf serum they were using in tissue culture medium contained factors that inhibited syncytium formation in HRSV-infected cells. Subsequent studies established that these inhibiting factors were specific antibodies and thus indicated that the same or a closely related virus must be present in cattle (Doggett et al., 1968). This apparently set the stage for isolation of a respiratory syncytial virus from cattle.

At the beginning of February 1967, an acute febrile respiratory disease was observed in cattle on several farms in the canton of Geneva, Switzerland. In mid-February, the epizootic disease spread to the cattle of three of the five farms of the small village of Avusy. Paccaud and Jacquier (1970) published a report of this outbreak. All animals less than seven years of age on two farms showed clinical signs of an acute respiratory disease. Interestingly, older cattle remained apparently healthy although they were in close contact with the sick animals. Although the first symptoms of the disease appeared serious, the course was relatively benign. It seems
worth mentioning that no respiratory or feverish disease was observed in the human beings who cared for the cattle, or who lived on the affected farms, or who visited them before or soon after the outbreak.

During the course of the investigation twelve specimens (conjunctivo-nasal swabs) were collected from calves and heifers on two farms. Two viruses were isolated one each from a calf and a heifer on the two farms. It soon became apparent that the isolates were identical and, furthermore, that they manifested many biological characteristics similar to HRSV isolates. On the second passage in primary bovine embryonic kidney 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 to develop rapidly in 5-6 days.

The physical and chemical properties of these bovine isolates closely resembled those of human isolates. They were found to be very heat labile and sensitive to low pH and lipid solvents. Serological studies were conducted with acute and convalescent serum samples of infected cattle. There was seroconversion in both affected herds, including the two animals from which the viruses were isolated. This was accepted as evidence for a bovine origin of the isolates and that these isolates were involved in the respiratory outbreak. Cross-neutralization tests with the bovine isolates and a human RS virus using guinea pig antiserum were also conducted. The results indicated that the bovine and human viruses were antigenically closely related
but not identical. Further serological studies revealed that one-third of all bovine sera tested had serum neutralizing antibodies to the bovine isolates. One of the bovine isolates was neutralized by over 50 percent of the human sera 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, Inaba et al. (1970) described a respiratory epizootic that swept through cattle in Japan during 1968 and 1969. They clearly documented that bovine respiratory syncytial virus was involved in this outbreak and that it was antigenically closely related to HRSV. In the same year Wellemans et al. (1970), reported isolation of BRSV from cattle in Belgium. 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 BRSV (Wellemans et al., 1971). In 1971 Jacobs and Edington reported isolation of BRSV from cattle in England. A few years later Koves and Bartha (1976) reported isolation of BRSV from calves in Hungary.

A serological study with various animal species was conducted to detect serum antibodies to BRSV in Canada (Berthiaume et al., 1973). Although it was a limited survey, there was an indication of antibody titers not only in cattle, but also in sheep. It was concluded from this study that, since a high percentage of sheep did have RSV antibodies, sheep might serve as an experimental model for the study of HRSV. Serum antibodies have also been reported in fallow deer in Italy (Giovannini et al.,
1988); white-tailed deer, mule deer, and hunter killed pronghorns in the United States (Johnson et al., 1986); Peruvian alpaca (Rivera et al., 1987), and goats in Zaire (Jetteur et al., 1990). Caprine respiratory syncytial virus has been isolated from a herd of pygmy goats (Smith et al., 1979). Serum antibodies have also been detected in cats, dogs and pigs (Baker and Frey, 1985).

The first isolations of BRSV from cattle in the United States were reported in Iowa and Missouri in 1974 by Smith et al. (1974) and Rosenquist (1974) respectively. Since then respiratory tract disease associated with BRSV has been reported from many countries. Serological surveys in Morocco (Mahin et al., 1985), Canada (Moteane et al., 1978; Elazhary et al., 1980; Lynch and Derbyshire 1986; Donkersgoed et al., 1993), Germany (Pernthaner et al., 1990), and Turkey (Burgu et al., 1990) have indicated the prevalence of BRSV to be 70.4%, 35.9% to 95%, 17.4%, and 46.12%, respectively. Virus isolation and serological studies have provided evidence that BRSV causes respiratory disease outbreaks in cattle worldwide.

Infection with BRSV apparently is common, as indicated by a high percentage of cattle with serum antibodies to BRSV in the United States. Antibody prevalence to BRSV has been reported to be 81% in Iowa (Smith et al., 1975), 38% in Maryland (Mohanty et al., 1975), 67% in Alabama (Rossi and Kiesel, 1974), 76.3% in Oklahoma (Potgieter and Aldridge, 1977) and 65.5% in Minnesota (Baker et al., 1985).
It has become apparent that BRSV plays a significant role in the bovine respiratory disease complex. Serum antibodies are not only high in cattle but the limited literature indicates considerable prevalence in sheep as well. A flock of 373 in Minnesota were found to have 52.5% seropositive ewes (Goyal et al., 1988). The respiratory syncytial virus has also been isolated from sheep (Smith et al., 1974; LeaMaster et al., 1983)

Pathogenesis

The pathogenic mechanisms involved in BRSV infections remain poorly defined and not well understood. Infected cattle transmit BRSV to susceptible cattle by aerosolized secretions and the virus gains entrance into a new host through the respiratory tract (Kahr, 1981). The BRSV replicates in the cells of the nasal mucosa, pharynx, trachea, and lungs of the calves exposed to experimental infection by the respiratory route (McNulty et al., 1983; Thomas et al., 1984; Castleman et al., 1985a). However, Trigo et al. (1984) detected viral antigen in the epithelial cells of the bronchioles as well as alveoli but they could not demonstrate viral antigen in the cells of nose, trachea, and bronchi. Viral antigen has also been demonstrated in alveolar macrophages (Castleman et al., 1985a). Trigo et al. (1985) reported abortive BRSV infections of bovine pulmonary alveolar macrophages in vitro. Scanning electron microscopy has demonstrated destruction of ciliated respiratory epithelium in calves 8-10 days after experimental infection (Eis, 1979). These
findings indicate that pulmonary clearance may be compromised by BRSV allowing secondary pulmonary infection by agents present in the upper respiratory tract.

BRSV and Pasteurella hemolytica have been shown to have synergistic pathogenic effects under experimental conditions in lambs (Al-Darraji et al., 1982; Trigo et al., 1984). Calves infected with lungworms had more severe clinical signs after infection with BRSV than non-parasitized calves (Verhoeff et al., 1988).

Although there are reports of respiratory disease outbreaks in cattle in which the only virus identified was BRSV, other investigators have reported concurrent infection with other viruses such as bovine virus diarrhea (BVD), infectious bovine rhinotracheitis (IBR), parainfluenza virus 3 (PI3) and adenovirus type 3 (Bryson et al., 1979; Elazhary et al., 1980; Johnson et al., 1981; Bryson et al., 1983; Steinhagen and Heckert, 1988). Lehmkuhl and Gough (1977) speculated that BRSV infection may facilitate infection by other viruses.

A biphasic pattern of clinical signs and the response to treatment with corticosteroids and antihistamines has given rise to a speculation that hypersensitivity may be involved in pathogenesis of BRSV infections in cattle (Bohlender et al., 1982; Frey, 1983). Epizootic evidence does not support this hypothesis since during the seasonal occurrence of the disease only calves not present during previous epizootics are affected (Verhoeff et al., 1984). Furthermore, experimental infection of calves followed by superinfection 10 days later did not appear to cause a severe disease; rather the calves appeared to be resistant to the second infection (Ciszewski et al.,
1987). The role of IgE antibodies in the pathogenesis of BRSV has yet to be determined. Limited studies indirectly implicate IgE antibody in the pathogenesis of BRSV. Stewart and Gershwin (1989) reported that, when calves previously vaccinated were challenged with the virus, there was a strong correlation between disease signs and histamine concentration in nasopharyngeal exudate. They also reported a correlation between signs of the disease and BRSV-IgE antibody concentration in the lung lavage fluid; this was true for the vaccinated group but not for the unvaccinated group. Plasma concentrations of histamine have also been reported to be significantly greater in calves infected with BRSV than in uninfected calves (Gershwin and Giri, 1992).

Clinical Features

The hallmark of RSV infections is their recurrence and variability in clinical signs and disease (McIntosh and Chanock, 1990). The virus is a major cause of lower respiratory tract disease in cattle and humans. In both species major problems due to RSV occur during the first six months of life when maternal immunity is still present (Stott and Taylor, 1985).

The cattle outbreak in Japan which affected 40,000 animals was characterized by anorexia, depression, respiratory distress, cough, nasopharyngeal secretions, and foamy with sometimes blood-stained saliva. The mortality rate in this outbreak was 0.26% (Inaba et al., 1970). Paccaud and Jacquier (1970) reported an outbreak in
cattle that developed rapidly in Switzerland. In most cases, only febrile involvement of the upper respiratory tract with nasal discharge and cough was noted. In about one-third of the cases however, a relatively high fever up to 41.5°C developed and discrete signs of bronchopneumonia were observed. A surprising observation was that the most severely ill animals were cows (aged less than 7 years) and not calves. Older cattle remained apparently healthy although they were in close contact with the sick animals. The disease subsided in 3 to 5 days in calves and heifers and 8 to 10 days in cows. There were no deaths or serious complications.

BRSV affects all types of cattle including adults (Inaba et al., 1970; Paccaud and Jacquier, 1970), weaned calves (Smith et al., 1975; Frey, 1983; Gillette and Smith, 1985) and calves (Rosenquist, 1974; Pirrie et al., 1981; Elazhary et al., 1982). The severity of the disease varies considerably from group to group, and within groups. Clinical signs in weaned calves have been described (Bohlender, 1981; Bohlender et al., 1982). Early signs included anorexia and a rapid increase in the frequency and severity of coughing. Respiratory signs were most prominent and included nasal and lacrimal discharges and increased respiratory rate. Elevated rectal temperatures ranged from 40-42.2°C. The authors reported that the early stage of the disease is difficult to detect since calves with increased body temperature often brighten up when approached. If the early stage is missed the disease can progress rapidly and calves may be found dead. Later stages of the disease are characterized by marked dyspnea, with mouth breathing, frothing of saliva and a dry hacking cough.
Subcutaneous edema was first observed as a slight puffiness around the eyes, and as it became more severe there was swelling in the mandibular area, throat latch and neck. Diarrhea has been reported but tends to be transient and may be followed by constipation because of decreased water and feed intake. Morbidity in a herd is usually high and duration of the disease may range from 10-14 days. Mortality is variable and may range from 0% to 20% (Holzhauer, 1979). If the disease is not recognized promptly while in the early stage, mortality may increase. Secondary bacterial pneumonia is a common occurrence in association with BRSV infections.

Subclinical and asymptomatic BRSV infections have also been reported (Rossi and Kiesel, 1974; Bohlender et al., 1982). In some cattle herds two stages of the disease have been described. The first stage is expressed as a mild respiratory disease characterized by nasal and lacrimal discharge followed by quick recovery. The second stage follows in several days to several weeks with a sudden onset of severe dyspnea. Death occurring in the second stage is associated with atypical interstitial pneumonia (Wellemans et al., 1978; Baker and Frey, 1985).

Most of the time experimental infections have been unsuccessful in producing a disease process characteristic of natural disease in calves. Experimental infections have resulted in mild respiratory disease characterized by fever, rhinitis and sometimes nasal discharge and cough (Eddington and Jacobs, 1970; Mohanty et al., 1975; Smith et al., 1975; Thomas et al., 1984; Castleman, et al., 1985a). However, Bryson et al. (1983) were successful in producing a severe respiratory tract disease
characterized by coughing, tachypnea, and hyperpnea in young calves. They gave the virus to calves by a combined intranasal (i/n) and intratracheal (i/t) route for four consecutive days. Similarly Ciszewski et al. (1991) induced severe respiratory tract disease in one-month-old calves that were colostrum-fed and seropositive to BRSV. They also administered the virus by a combined respiratory tract route (i/n, i/t) for four consecutive days. Although ovine respiratory syncytial virus has been isolated from sheep, widespread outbreaks have not been reported. Experimental inoculation of lambs with BRSV causes a mild clinical response consisting of a transient pyrexia, hyperpnea, slight nasal discharge, coughing and dullness (Al-Darraji et al., 1982; Trigo et al., 1984; Sharma and Woldehiwet, 1990, 1990a, 1992).

Characteristics of Respiratory Syncytial Virus

Morphology: The study group on Paramyxoviridae, Vertebrate Virus Subcommittee of the International Committee on Taxonomy of Viruses has placed the three genera Paramyxovirus, Morbillivirus and Pneumovirus in the family Paramyxoviridae. The genus Pneumovirus includes HRSV (type species), BRSV, and pneumonia virus of mice. Recently, turkey rhinotracheitis virus has also been placed in the genus Pneumovirus (Pringle, 1991). According to the Committee the overall shape of HRSV is pleomorphic, usually roughly spherical, but filamentous forms are frequent. Pleomorphic and spherical forms are 80-500 nm in diameter and filamentous forms are 60-110 nm in diameter and 5 μm in length. The virus particles have a 7-15 nm
thick envelope covered with 10-12 nm projections (Kingsbury et al., 1978). Armstrong et al. (1962) reported that filamentous forms have a uniform width of 60-70 μm, and up to 2 μm in length and that they are bounded by a layer of exceptionally high density. They further concluded that transection of filaments would naturally account for at least some of the circular profiles in the micrographs; thus truly spherical particles amounted, at most, to a minority of those actually seen. Other workers (Norrby et al., 1970; Bachi and Howe, 1973; Berthiaume et al., 1974) have also reported the virus particles of HRSV to be pleomorphic with a variable diameter of 100-350 nm. In addition to the pleomorphic forms, filamentous forms were also frequently observed with dimensions of 60-110 nm diameter and up to 5 μm length. The filamentous forms predominated. Berthiaume et al. (1974) also suggested that the spherical to oval forms are cross and oblique sections of the filamentous forms. The lipid bilayer membrane encloses an RNA nucleocapsid which contains a nonsegmented, negative-strand RNA genome (Huang and Wertz, 1982) and the virus matures while budding through the cell membrane (McIntosh and Chanock, 1990).

Ito et al. (1973) studied BRSV in calf kidney cell cultures and reported that in negatively-stained preparations the virus particles showed great pleomorphism. Many virus particles appeared roughly spherical, others were more or less elongated or distorted. The overall diameter varied from 80-450 μm and intact virions had a thick membrane (70-150 Å), covered with mostly club-shaped projections, measuring 130-
170 Å in length. In ultrathin sections spherical particles measuring 80-130 mµ in diameter along with filamentous forms of 100-130 mµ in diameter with variable length were seen. They also observed dots with a diameter of 11-15 mµ, as previously reported by Norrby et al. (1970) in HRSV, within the budding virus particles. Based upon the structure, as well as the site and mode of maturation of BRSV, HRSV, and pneumovirus of mice they proposed that these three be grouped together as metamyxoviruses.

Moussa (1994) has also reported that the BRSV particles are filamentous in form, about 80-120 nm in diameter, and variable in length. Belanger et al. (1988) studied the structure of BRSV grown in a continuous cell line of ovine kidney (OK) origin and reported the presence of well-defined bridges between the virus particles. Similar bridges were also observed between BRSV particles grown in Vero cells or bovine kidney cells. In contrast no such bridges were apparent between HRSV particles (Long strain) grown under similar conditions. They further reported that the BRSV particles, like HRSV, appeared to be surrounded by an 7.5 ± 1.7 nm electron-dense membrane and were mainly filamentous forms. Their length varied considerably, with values reaching up to several micrometers, but the diameter was relatively constant at 94.6 ± 5 nm.

Genomic organization: The genome of HRSV is a single negative-sense strand of RNA which is composed of 15,000 nucleotides (Collins et al., 1987). It contains one promoter and ten genes that are transcribed as a single unit from the promoter at the
3' end of the genome (Dickens et al., 1984). Ten unique mRNAs are transcribed and each codes for a unique protein (Huang et al., 1985). The order of the transcription is 3' 1C-1B-N-P-M-1A-G-F-22K-L 5' (Collins et al., 1987). The HRSV genes are nonoverlapping except for the last two genes in the 3'–5' order. This overlapping by 68 nucleotides results in down regulation of L protein synthesis (McIntosh and Chanock, 1990). Like HRSV the BRSV genome also encodes for ten different mRNAs. The electrophoretic pattern of BRSV mRNAs is similar to that of HRSV (Lerch et al., 1989; Mallipeddis et al., 1990).

Viral proteins: Extensive experimentation has been conducted to define the molecular biology of HRSV. Ten viral proteins (Table 1) have been identified: large (L), glycoprotein (G), fusion (F), nucleocapsid (N), phosphoprotein (P), matrix (M), 14K (1B), 22K (M2), 15K (1C), and 9.5K (1A). Two of the proteins (1B,1C) are nonstructural and the remaining eight are structural. The G, F, 1A, and 22K proteins are associated with the membrane of infected cells and the virus envelope. The G, F, and 1A are glycosylated (Kimman and Westenbrink, 1990). The 22K protein is not glycosylated and its exact location in the virion is not known (McIntosh, 1978); it may be a second matrix protein (M2). The functions of 1A and 22K are not known (McIntosh and Chanock, 1990).

The G protein has a high carbohydrate content, as is indicated by the fact that its protein moiety is relatively small (32.6 Kd) in relation to the mature fully processed
Table 1. Structural and nonstructural proteins of HRSV, strain A₂ of subgroup A

<table>
<thead>
<tr>
<th>Protein/designation</th>
<th>MW (kilodaltons)</th>
<th>Location</th>
<th>Function</th>
<th>Additional known properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS1 (1C)*</td>
<td>15.6</td>
<td>Not in virion</td>
<td>Not known</td>
<td>Slightly acidic</td>
</tr>
<tr>
<td>NS2 (1B)*</td>
<td>14.7</td>
<td>Not in virion</td>
<td>Not known</td>
<td>Basic</td>
</tr>
<tr>
<td>N</td>
<td>43.5</td>
<td>Nucleocapsid</td>
<td>Structural protein of nucleocapsid</td>
<td>Abundant; tightly complexed with genomic RNA</td>
</tr>
<tr>
<td>P</td>
<td>27.1</td>
<td>Nucleocapsid</td>
<td>Component of polymerase complex?</td>
<td>Phosphorylated; relatively acidic</td>
</tr>
<tr>
<td>M</td>
<td>28.7</td>
<td>Inner aspect of viral envelope</td>
<td>Inner lining of viral envelope</td>
<td>Relatively basic, moderately hydrophobic</td>
</tr>
<tr>
<td>SH (1A)*</td>
<td>15 and 21-30</td>
<td>Surface of infected cell</td>
<td>Not known</td>
<td>Strongly hydrophobic core of 28 amino acids.</td>
</tr>
<tr>
<td>G</td>
<td>84-90</td>
<td>Surface of viral envelope</td>
<td>Attachment to host cell receptors</td>
<td>High content of carbohydrate, 90% of which is O-linked to serine and threonine.</td>
</tr>
<tr>
<td>F</td>
<td>68-70</td>
<td>Surface of viral envelope</td>
<td>Fusion of viral envelope with host-cell envelope; syncytium formation</td>
<td>Cleaved proteolytically to yield disulfide linked F₂ (20 kd) and F₁ (48 kd).</td>
</tr>
<tr>
<td>M2 (22K)*</td>
<td>22.2</td>
<td>Viral envelope</td>
<td>Not known</td>
<td>Hydrophilic and very basic.</td>
</tr>
<tr>
<td>L</td>
<td>250</td>
<td>Nucleocapsid</td>
<td>Polymerase of nucleocapsid?</td>
<td>Low abundance in virions and infected cells</td>
</tr>
</tbody>
</table>

*Former designations.
Source: McIntosh and Chanock, 1990.
protein (84-90 Kd) (Wertz et al., 1985). It mediates attachment of the virion to the cell membrane (Levine et al., 1977). The F (68-70 Kd) protein is glycosylated, and is composed of two disulfide-linked subunits, \( F_1 \) and \( F_2 \) (Dubovi, 1982; Lambert and Pons, 1983; Huang et al., 1985). The F protein mediates fusion between the cell membrane and the viral envelope. Many monoclonal antibodies that precipitate this protein inhibit cell to cell fusion caused by RSV (Walsh and Hruska, 1983). Three proteins N (43.5 Kd), P (27.1 Kd), and L (250 Kd) are located in the RSV nucleocapsid. N is an abundant protein that serves a structural function, whereas the other two probably play roles in transcription and replication of viral RNA that remain to be defined (McIntosh and Chanock, 1990). The 28.7 Kd M is present in detergent-solubilized cores but not in nucleocapsids, which suggests that it is analogous to the matrix protein of other RNA viruses (McIntosh and Chanock, 1990).

In contrast, until recently very little was known about the molecular biology of BRSV. In a preliminary report Cash et al. (1977) compared the polypeptides of HRSV and BRSV and demonstrated six BRSV-specific polypeptides that migrate very closely to F, N, P, M, 24K and 10K proteins of HRSV. Lerch et al. (1989) identified nine polypeptides of BRSV in infected cells by comparing the proteins of BRSV- and HRSV-infected cells and by immunoprecipitation and western immunoblot assays using BRSV-specific antiserum. Mallipeddi et al. (1990) recently identified and characterized all ten virus-specific polypeptides (Strain A51908) ranging in molecular weight from approximately 200 to 11K (Table 2). Viral
polypeptides were detected as early as 30 min postinfection and their synthesis reached a plateau 12h after infection. The P protein was highly phosphorylated and G, F were glycosylated. N, P, and M were found to be the major polypeptides in cells infected by the virus.

Table 2. Molecular weights of viral-induced proteins in BRSV infected cells

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>Molecular weight (Kd)</th>
<th>Post-translational Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>200</td>
<td>-</td>
</tr>
<tr>
<td>G</td>
<td>85-100</td>
<td>Glycosylated</td>
</tr>
<tr>
<td>F</td>
<td>68</td>
<td>Glycosylated</td>
</tr>
<tr>
<td>-F₁</td>
<td>48</td>
<td>Glycosylated</td>
</tr>
<tr>
<td>-F₂</td>
<td>20</td>
<td>Glycosylated</td>
</tr>
<tr>
<td>N</td>
<td>42</td>
<td>-</td>
</tr>
<tr>
<td>P</td>
<td>34</td>
<td>Phosphorylated</td>
</tr>
<tr>
<td>M</td>
<td>29</td>
<td>-</td>
</tr>
<tr>
<td>23K</td>
<td>23</td>
<td>-</td>
</tr>
<tr>
<td>15K</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>13.5K</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>11K</td>
<td>11</td>
<td>-</td>
</tr>
</tbody>
</table>

Source: Mallipeddi et al., 1990.

Subgroups: Comprehensive studies of the HRSV isolates using monoclonal antibodies, polyclonal antibodies, immunizing experiments in cotton rats, and monitoring the immune response following natural infection in infants and children have delineated at least two antigenic variants designated as subgroup A and B or 1
and 2. The antigenic differences between the subgroups have been found to be more pronounced with G protein than other proteins (Mufson et al., 1985; Hendry et al., 1986, 1988; Johnson et al., 1987). Variation even within the groups has also been reported. Recently six subdivisions have been identified for subgroup A and two to four groups of antigenic variance have been identified within the subgroup B (Akerlind et al., 1988; Anderson et al., 1991). Although both subgroups are reported to cocirculate during respiratory outbreaks, their prevalence varies both during outbreaks and from outbreak to outbreak, but subgroup A generally prevails (Hendry et al., 1986a; Mufson et al., 1988; Russi et al., 1989). However a recent report regarding subgroup analysis of 613 specimens from Vancouver, Canada revealed that from 1987-1992 viruses of subgroup A prevailed (60% to 80% of isolates) except during 1988-1989 when subgroup B viruses represented up to 94% of the isolates (Thomas et al., 1994). Respiratory disease caused by viruses of each group seems not to differ clinically (Heilman, 1990).

Until recently, BRSV was considered to be a monotypic virus because there have been limited attempts to determine the occurrence of antigenic variation among BRSV strains. The G protein of BRSV has been reported to possess major antigenic differences from the HRSV G protein by immunoprecipitation and western blot analysis; however the F, N, M and P proteins of BRSV had antigenic cross-reactivity with their HRSV counterparts (Lerch et al., 1989). Furthermore, the G protein of BRSV was recognized only by a polyclonal antiserum against the homologous BRSV
strain (391-2). The inability of the polyclonal antiserum raised against BRSV strain 127 to recognize the G protein of BRSV strain 391-2 indicated that BRSV has antigenic subgroups like HRSV (Lerch, et al., 1989). Baker et al. (1992) studied the proteins of HRSV and BRSV in detail and reported major variations in the molecular sizes of the P and F proteins of nine BRSV isolates. The SBAH, MN, and 375 strains had a P protein of smaller molecular size (36Kd) than the other six strains which expressed a 38Kd P protein. They further reported the molecular size of F₀, F₁, and F₂ as 68, 48, and 21 Kd for SBAH strain and 63, 47, and 17 Kd for the remaining BRSV strains respectively. Based on their findings they raised a question about the efficacy of strains used to prepare current BRSV vaccines in the United States and also speculated that the vaccine failures may be due to the antigenic differences among BRSV isolates.

Viral target antigens: Commercial and experimental BRSV vaccines have been studied for efficacy in stimulating antibody as well as cell-mediated responses. There is a single report where immunoaffinity purified F protein of BRSV has been used for inducing an immune response (Nelson et al., 1992). These authors concluded that seronegative calves responded to primary vaccination with immunoaffinity purified F protein by producing neutralizing antibodies. However, subsequent vaccination did not increase the antibody response which suggested that the already existing immunity suppressed local and systemic neutralizing antibody responses.
Almost all of the viral proteins of HRSV, including structural and non-structural, have been studied for their role in providing protection against infection in experimental animals. In a variety of immunization experiments the F and G proteins have been found to provide resistance against HRSV experimental infections; this indicates that these glycoproteins are most probably the major independent protective antigens (McIntosh and Chanock, 1990). Viral proteins used in such experiments have either been immunopurified or expressed in vaccinia virus.

Passive transfer of monoclonal antibodies raised against F and G proteins significantly reduced or completely prevented replication of HRSV in the lower respiratory tract of mice and cotton rats (Walsh et al., 1983). Walsh et al. (1987) reported that cotton rats immunized with purified G or F protein (reduced or nonreduced) developed complete pulmonary resistance to HRSV challenge. However, the nasal passages were only partially protected. The animals immunized with either reduced or nonreduced F protein developed neutralizing as well as fusion inhibiting antibodies. G protein induced only neutralizing antibodies.

Levine et al. (1989) evaluated a lyophilized subunit vaccine containing F, G, and M proteins in SJL mice. A single dose of this vaccine (50 μg) induced antibodies to G which lacked neutralizing activity. Mice given two doses of the same vaccine (50 μg and 25 μg) produced antibodies to all of the three proteins and the antisera possessed neutralizing activity for RSV. All the vaccinates were protected from intranasal challenge.
Nicholas et al. (1990) and Openshaw et al. (1990) reported that cytotoxic T lymphocyte (CTL) recognition of cells expressing only 22-Kd protein of RSV was comparable to CTL recognition of virus-infected cells. The recognition of F and N protein was comparatively less. This prompted Nicholas et al. (1991) to explore the role of 22-Kd protein in the anti-RSV immune response and its role in providing protection against RSV infections. They utilized recombinant vaccinia viruses expressing the F protein (Vac-F), FG (Vac-FG), consisting of extracellular domains of both F and G), or 22-Kd (Vac-22Kd) protein, for their ability to induce humoral, CTL responses and protection in BALB/c mice against A and B subgroups of RSV. Immunization with Vac-F induced very high titers of RSV-specific antibodies in all of the mice. Immunization with Vac-FG also induced a high titer of RSV-specific antibodies, but the titer was substantially lower than that induced by Vac-F. Immunization with Vac-22-Kd did not induce any detectable antibody to RSV in 88% of the vaccinates. The remaining 12% developed very low titers, which overlapped with the negative control values. Vac-F and Vac-FG also induced significant levels of cytolytic activity for RSV-infected cells and provided full protection against challenge with viruses of either subgroup of RSV. Immunization with Vac-22Kd induced particularly high levels of cytolytic activity for RSV-infected cells and provided significant protection against both subgroups of RSV. Based on their findings Nicholas et al. (1991) proposed that, in vivo, 22-kd-specific CTL can
provide at least partial resistance to RSV replication within the lung in the absence of a significant humoral component.

The N protein has been identified in several studies as a minor protective antigen. It probably plays a role in protective immunity through its participation in inducing CTL responses (McIntosh and Chanock, 1990). King et al. (1987) reported that N protein expressed in vaccina virus afforded a significant degree of protection in BALB/c mice. Although vaccination with the recombinant virus expressing N protein resulted in a significant reduction in titers in the lungs of mice following live-virus challenge, this protection was not as solid as provided by F and G proteins expressed and used similarly (Stott et al., 1986, Wertz et al., 1987). Similarly, rats vaccinated with a vaccinia recombinant virus that expressed the N protein were not as well protected as those immunized with vaccinia recombinants expressing F or G proteins (Kimman and Westenbrink, 1990). Bangham et al. (1986) has demonstrated that the N protein is one of the target antigens for CTL in man and mouse. Connors et al. (1991) have reported that viral proteins F, G, M2 (22-Kd), and N expressed in vaccinia virus provide protection to BALB/mice against RSV challenge, but the protection induced by M2 and N is comparatively short-lived. They monitored the resistance by challenging the mice i/n with RSV on days 9 or 28 postvaccination. The F and G proteins provided complete protection on both days. M2 and N induced significant but not complete resistance on day 9, but little protection by day 28.
Immunization with other viral proteins, also expressed in vaccinia virus, including P, SH (1A), M, 1B, and 1C did not induce detectable resistance to RSV challenge.

Trudel et al. (1989) have indicated that immunostimulating complexes (ISCOMS) made from F and N proteins of RSV induce neutralizing antibodies in guinea pigs. Similarly BALB/c mice have been reported to be protected from live virus challenge by ISCOMs, composed primarily of F, N and, to a lesser extent, G proteins (Trudel et al., 1991). Peretz et al. (1992) reported that immunopurified F, G, but not N protein induced protection in BALB/c mice. The N protein did not block challenge infection but F and G caused a 30 fold reduction of virus titer in lung tissue. In a very comprehensive study Connors et al. (1992) demonstrated that antibodies induced by F and G proteins, expressed in vaccinia virus, were alone sufficient to provide protection to BALB/c mice, because depletion of T cells or interferon did not affect their high level of resistance to RSV challenge. However the resistance induced by M2 protein, also expressed in vaccinia virus, was mediated primarily by CD8+ T cells. The resistance induced by Vac-M2 was completely abrogated by depletion of CD8+ T cells, whereas depletion of CD4+ T cells or interferon had an intermediate effect on resistance to challenge. Similar findings have been reported by Kulkarni et al. (1993). Their results indicated that significant resistance could only be induced by Vac-M2 in BALB/c (H-2d haplotype) and not in BALB.K (H-2b) or BALB.B (H-2b) mice. This resistance was mainly mediated by pulmonary CTLs and that resistance waned to very low level in two months following immunization. A
surprising observation in their study was that none of the other RSV proteins studied, including P, N, M, 1C, 1B, and SH (1A), induced significant resistance in these three haplotypes of mice.

In a limited study, Stopner et al. (1993) investigated pairs of acute and convalescent sera, obtained from young children aged 1 month to 1 year with acute RSV infection, for antibody levels to the C-terminal peptide of SH (1A) protein. The antibody levels to the C-terminal were low suggesting that the SH protein was a weak stimulant of antibody production in children with natural infection. Recently Walsh (1993) has documented that inclusion of cholera toxin (CT) with immunopurified F protein enhances the resistance of Swiss mice to RSV challenge.

Diagnosis

The diagnosis of BRSV is challenging and can be made by several methods including virus isolation, immunofluorescence and serology. Virus isolation can be made from nasopharyngeal swabs, lacrimal discharges, tracheal washes and lung lavages. The latter two samples are ideal for isolation purposes (Kimman, 1986). Since the virus is labile, it may be inactivated when the sample is frozen at collection and thawed in the laboratory (Bohlender et al., 1982). Since multiple passages may be required to detect the virus, it is considered to be the least reliable method for diagnosis (Baker, 1993). Paccaud and Jacquier (1970) identified BRSV after 32 days in culture and Inaba et al. (1972) had to wait for 17 days. However, efficiency of
isolation is enhanced if the samples are inoculated onto susceptible cells at the time of collection (Mohanty et al., 1976). Similarly, Frey (1982) had greater success in isolation by inoculation of cell cultures immediately after sampling. Collection of samples from unaffected animals that are in close contact with the clinically sick animals may increase the chances of BRSV isolation (Kahrs, 1981).

Immunohistochemistry has been reported to be the most dependable method for positive identification of BRSV in infected tissues (Smith and Collins, 1987; Baker and Frey, 1985; Thomas and Stott, 1981).

The indirect immunofluorescence (IIF) test not only enables a diagnosis to be made very rapidly but also the titers by this method have been found to be higher than with a complement fixation test (Welleman, 1977). Anti-BRSV antibodies in serum, nasal mucus and organ extracts can be detected rapidly by this method. Fluorescent antibody staining of frozen lung tissue or nasal exudate is used in most diagnostic laboratories. Immunofluorescence testing gives better results with postmortem lung samples than with nasopharyngeal swabs (Thomas and Stott, 1981).

The most commonly used serological test is the microtitration serum-virus neutralization test (Baker, 1993). Caution is required if this test is to be performed on calves less than four months of age due to the potential presence of maternally-derived antibodies and the possibility of infection (Smith et al., 1975; McNulty et al., 1983). A serological diagnosis is not possible in calves that still have passive
immunity. A possible approach to overcome this problem is to sample calves representing various age groups (Baker, 1993).

**Immunity and Immunization**

Both HRSV and BRSV infections are common in infants and calves respectively and can cause severe disease (Stott and Taylor, 1985; Glezen et al., 1981; Harrison and Purseil, 1985). Infections in adults tend to result in less severe disease (Baker et al., 1986; Hall et al., 1978). Maternally-derived passive immunity does not provide complete protection in calves or infants (Baker et al., 1986a, 1986b, 1986; Glezen et al., 1981; Lehmkuhl et al., 1979; Rosenquist, 1974). Repeated infections occur in humans and cattle but the subsequent infections are comparatively less severe than the initial infections (Stott and Taylor, 1985; Baker et al., 1986; Glezen et al., 1981; Lehmkuhl et al., 1979; Rosenquist, 1974; Baker et al., 1985; Hall et al., 1976; Martin, 1983).

The role of maternal immunity has been a matter of great debate because HRSV and BRSV infections can occur in the presence of maternal antibodies. However, studies do indicate that, although passively derived maternal immunity does not prevent infection with HRSV, it may provide some protection in ameliorating the severity of the disease (Glezen et al., 1981; Ogilvie et al., 1981; Hall et al., 1978; Lamprecht et al., 1976). Similar findings have been reported in calves. An epidemiological study of BRSV demonstrated that, although maternally-derived
antibodies did not prevent infection in calves, the incidence and severity of the disease were inversely related to the level of specific maternal antibody (Kimman et al., 1988). Recently Belknap et al. (1991) reported that passively-derived antibody reduced the severity of the disease when calves were infected at 48 hours of age.

The precise role of immunity in RSV infections is not well understood. It has also been proposed that immunopathological mechanisms might play a role in RSV infections. This theory is speculative, since, if immunopathologic mechanisms are important in RSV disease pathogenesis, these need to be clearly defined (Baker and Velicer, 1991). It appears that serum antibody is perhaps the major mediator of immunity to natural infection with RSV. Cell-mediated immunity appears to be important in recovery from RSV infection because viral shedding persists for prolonged periods in individuals with a defect in cell-mediated immunity. However there is no evidence that the cell-mediated immunity plays any role in prevention of infection (Tristram and Welliver, 1993).

Currently there are no vaccines available in the United States to control the disease in humans, and efforts to develop a vaccine to protect infants and young children have thus far failed (Baker and Velicer, 1991). However both live and inactivated candidate vaccines for infants and children continue to be tested. In contrast, a modified-live virus vaccine for BRSV has been available in Europe since 1978. In the United States modified-live BRSV vaccines and killed vaccines became available in 1984 and 1988 respectively (Baker and Velicer, 1991). There are
conflicting reports on the possible effects of live and inactivated vaccination on the prevalence of BRSV infections. Several workers have reported that vaccinations reduced the prevalence and severity of the disease (Kubota et al., 1992, 1990; Howard et al., 1987; Stott et al., 1987, 1984; Morter and Amstutz, 1986; Thomas et al., 1986; Frennet et al., 1984) while others found no significant effect (Morisse et al., 1990; Ploeger et al., 1986; Holzhauer and Wertenbroek, 1979). A major limitation of these vaccines is that maternally-derived antibody to BRSV suppresses an active immune response in calves (Kimman et al., 1987; Westenbrink et al., 1989). Not only the humoral response but also the mucosal antibody response is suppressed; however, immunization via the respiratory tract did prime calves, having maternal immunity, for memory responses (Kimman et al., 1989; Kimman et al., 1987; Westenbrink et al., 1989). Thus it is imperative that RSV vaccines should have the ability to induce immunity or at least prime the subject in the presence of passive immunity because of the early age distribution of severe disease associated with RSV.

**Solid Matrix-Antibody-Antigen Complexes**

Antibody-antigen complexes are known to enhance the immune response and have been used for the induction of both humoral and cell mediated immune responses (Chang et al., 1985). Monoclonal antibodies (MAbs) can be attached to particulate solid matrices, and the solid matrix-antibody complexes can be used to purify the
required antigen. The resulting complexes, solid matrix-antibody-antigen complexes (SMAA), can be used as immunogens.

Most of the research work involving SMAA complexes as immunogen has been undertaken by Randall and his associates, mentioned below. The solid matrix they used for most experiments in experimental animals was a "fixed" and killed suspension of the Cowan A strain of *Staphylococcus aureus*.

A major advantage of designing solid matrix-antibody-antigen (SMAA) complexes as vaccines is in the preparation of the immunogen. Many other methods being developed to present antigens to the immune system first require the purification of the protein of interest, which often involves strong denaturing conditions that may irreversibly alter some antigenic determinants. To produce SMAA complexes a simple procedure of antigen purification can be used. Also, because the resulting SMAA complexes are used as the immunogen, there is no need to use strong denaturing conditions to remove the antigen from the complex. These complexes are non-infectious, and therefore there is no need to inactivate them using formaldehyde or heat. These procedures are commonly used in the production of whole killed virus vaccines and can selectively denature particular antigenic determinants and adversely affect the induction of a protective immune response. SMAA complexes are also relatively stable to prolonged storage conditions, a property that may be advantageous in developing countries where it may be difficult to store some vaccines (Randall, 1989).
SMAA complexes have been used, by incorporating in them either internal or external proteins from the same virus or from different viruses, for immunization as well as determining the humoral and cytotoxic T cell immune responses. Prior immunization of mice with SMAA complexes containing either surface or internal structural proteins of simian virus 5 (SV5) reduced the amount of virus replication within infected lungs; the greatest degree of protection was induced by nucleoprotein or matrix protein (Randall et al., 1988). In another study Randall and Young (1988) observed that immunization with SMAA complexes, containing internal or external structural proteins of SV5 without an adjuvant, induced higher levels of antibody than the antigen alone precipitated on alum. Further analysis of the cytotoxic T cell responses of immunized animals indicated that both surface and internal structural proteins can act as target antigens. Randall and Young (1991) further demonstrated that immunization with SMAA complexes can induce class 1 restricted cytotoxic T cells (CTLs).

SMAA complexes have also been constructed to produce multivalent immunogens containing various virus glycoproteins known to be involved in inducing protective immunity. Such complexes, containing glycoproteins of herpes simplex, HA protein of influenza and measles viruses, and HN protein of SV5 and parainfluenza virus type 2, induced powerful responses in mice. Serum analysis revealed high titers of antibodies to all of these viruses (Randall and Young, 1989).
In developing vaccines to counter viral infections like HRSV and BRSV, it is imperative to produce vaccines that engender the type of immune response best suited to provide protection and avoid immunopathology. Thus viral vaccines which, in addition to inducing neutralizing antibodies, can also play an important role in regulating T cell responses may fulfill these requirements.
INTRACYTOPLASMIC MATURATION AND BRIDGE FORMATION OF BOVINE RESPIRATORY SYNCYTIAL VIRUS STRAIN 375 IN VERO CELLS

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Summary. In the present study the morphology of bovine respiratory syncytial virus (BRSV strain 375) was observed in Vero cells at a stage when extensive cytopathic effects were present.

Electron microscopic examination of ultrathin sections of BRSV-infected Vero cells revealed not only spherical and filamentous forms of the virus but also bridges between the spherical particles. The length of bridges was 13.1 ± 1.6 nm from the outer surface of one particle to the other particle. The bridges did not appear to be arranged at a particular angle. Because of the bridges the virus particles occurred in large aggregates requiring that this factor be taken into account while purifying the virus. The diameter of filamentous forms was uniform but their length varied. Both forms were observed budding from the cell membrane of infected cells. Most of the observed mature particles were extracellular. However a few spherical particles with and without internal structure were also present in intracytoplasmic vesicles. This
confirms an earlier report on assembly of virus particles intracellularly in Vero cells infected by a member of the family Paramyxoviridae.

INTRODUCTION

The genus Pneumovirus of the family Paramyxoviridae and subfamily pneumovirinae currently contains human respiratory syncytial virus (HRSV), bovine respiratory syncytial virus (BRSV), ovine respiratory syncytial virus (ORSV), caprine respiratory syncytial virus (CRSV), pneumovirus of mice and turkey rhinotracheitis virus [5]. The members of the family are pleomorphic in structure, have an envelope derived from the host cell cytoplasm, and they mature by budding from the plasma membrane [8]. Virions observed by electron microscopy are either filamentous or spherical in appearance [8].

The structure of BRSV strain NMK 7 has been described [7]. Observations of ultrathin sections revealed spherical particles measuring 80-130 μm in diameter, and also filamentous forms of 100-130 μm in diameter of variable length. Belanger et al. [2] reported well-defined bridges between BRSV particles (strain A 51908), measuring 12 ± 2.7 nm. A prominent feature of these bridges was their arrangement at a 60° angle. No such bridges were reported in strain NMK 7 by Ito et al. [7]. Until recently BRSV was considered to be a monotypic virus since there had been limited attempts to determine the antigenic variation among BRSV strains.
However, very recent studies indicate that like HRSV, BRSV may also be divided into two antigenic groups [1, 6]. Furthermore, the hallmark characteristic of the members of the Paramyxoviridae family is independent assembly of their nucleocapsid within the cytoplasm and release of the enveloped viruses by budding through the plasma membrane [12]. But recently, Moussa [13] reported intracytoplasmic maturation of a strain of BRSV. In the present investigation, BRSV strain 375, originally isolated from nasal secretions of a calf [9], was observed by electron microscopy in ultrathin sections of infected Vero cell cultures. The present investigation provided additional evidence of intracytoplasmic maturation of BRSV and the presence of bridges between mature virions.

MATERIALS AND METHODS

Cells

Vero cells (Veterinary Diagnostic Laboratory, College of Veterinary Medicine, ISU., CRL 1587 Vero 76 from the American Tissue Culture Collection) were maintained on Dulbecco's Modified Eagles's Medium [(DMEM), Sigma Chemical Company, St. Louis] supplemented with 10% betapropiolactone-treated fetal calf serum (Sigma) and 50 μg/ml of gentamicin (Schering Corporation, Kenilworth, NJ).
Propagation of Virus

Vero cells grown in 175 cm² tissue culture flasks (Costar Corporation, Cambridge, MA) were inoculated with BRSV strain 375 when the monolayer was subconfluent and incubated in a humidified chamber with 5% CO₂ at 37°C. A low titer of virus i.e., 10⁹/ml was obtained up to day 3 to 4 postinfection. Modification of the culture procedure resulted in a methodology that routinely yielded a titer of 10⁶.⁵/ml.

Cells were infected with virus (2x10⁹/ml) when the monolayer was about 80% complete. When viral cytopathic effect (CPE) was initially observed, usually 44-50h postinfection, the cells were trypsinized and transferred with an equal quantity of trypsinized uninfected Vero cells to a 225 cm² flask (Corning Incorporated, Corning, NY). On further incubation (24-48h), 90-100 % CPE was present and the cells were harvested for electron microscopy.

Immunofluorescent Assay

Infected and uninfected Vero cells (2x10⁴ cells/well) were grown on teflon coated slides (ICN Biomedicals, Aurora, Ohio). When CPE was distinct, the cells were fixed in 80% acetone at room temperature and stored at -20°C. A monoclonal antibody (MAb) raised against BRSV (courtesy Professor Dr. Kenneth B. Platt, MIPM, ISU) or negative supernatant from SP2/O cells were added to the wells. Bound antibody was detected by probing with fluorescein isothiocyanate (FTTC)-
labeled anti-mouse IgG [Kirkegaard & Perry Laboratories, Inc. (KPL), Gaithersburg, MD] diluted 1:40 in PBS. Processed slides were examined under ultraviolet light for fluorescence.

**Electron Microscopy**

Cells were harvested when extensive CPE had developed. The medium was discarded and the monolayer was washed with phosphate buffered saline (PBS, pH 7.2) followed by the addition of 3% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.2). The cells were fixed for ten min, removed from the flask surface with a cell scraper, and centrifuged at 1500 G for ten min. Uninfected Vero cells were prepared by the same method. Cell pellets were resuspended in 3% glutaraldehyde for two hours at 4°C. The cells were subsequently washed in the 0.1 M cacodylate buffer three times, post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for two hours, dehydrated in a graded acetone series, and embedded in epoxy resin (Embed 812, Electron Microscopy Science, Fort Washington, PA). Ultrathin sections were cut on an LKB ultramicrotome, stained with 2% uranyl acetate and Reynold's lead citrate and examined with a Hitachi H500 transmission electron microscope.

Electron micrographs were taken on Kodak Electron Microscope Film 4489 (Eastman Kodak Company, NY) using a two second exposure time. The film was developed in Kodak D-19 developer diluted 1:2 with distilled water for four min at
42°F. Prints were made using a Durst Labrator 138-S enlarger with a point light source. The paper used for this purpose was either Kodabromide grade 4 or 5 or Kodak Polycontrast III RC with Kodak Polycontrast filter number 4 or 5. It was developed 1-2 min in Kodak Dektol Developer diluted 1:2 with distilled water, fixed in Kodak fixer and dried with an Omega/Akray DualDri model 150 print dryer.

RESULTS

Light Microscopy

The extensive cytopathogenic changes induced by BRSV are illustrated in Fig. 1. Large syncytia containing over 30-50 cells were generally seen.

Immunofluorescence Assay

The presence of the BRSV in the infected cells was demonstrated by indirect immunofluorescence assay. Infected cells stained with indirect immunofluorescence method using a MAb specific for BRSV and FITC labeled anti-mouse IgG demonstrated typical diffuse bright apple green color (Fig. 2). Uninfected cells failed to fluoresce.

Electron Microscopy

In the ultra thin sections of the virus-infected Vero cells, maturation of virus particles was observed at the cytoplasmic membrane (Fig. 3). Five to seven dots,
Figure 1. Vero cells infected with BRSV. Large unstained syncytia can be seen all over the microscopic field. X250

Figure 2. BRSV antigen in acetone fixed infected Vero cells stained by indirect fluorescent antibody technique. X400
representing the nucleocapsid, appear beneath the membrane of individual virus particles half way through their budding-off process. The virus particles displayed pleomorphism with spherical particles measuring 102.4 ± 7.8 nm in diameter (Fig. 4,5) and filamentous forms 74-109 nm in diameter and of variable length.

Both filamentous and spherical particles were either empty or contained electron dense internal components. The filamentous as well as spherical forms without inner structure outnumbered the forms having internal structure. Two parallel strands with a diameter of 7-10 nm were detected in the core of some filaments (Fig. 5). The nucleocapsid of many spherical particles was represented by "dots" numbering 10-12 per particle (Fig. 4). Some particles had fewer dots. The dots were found to be of very uniform size and their diameter was 15.1 ± 0.8 nm.

Well defined structures forming bridges between spherical particles were regularly seen. Their length was 13.1 ± 1.6 nm (Fig. 5) from the outer surface of one particle to the other particle. In some cases they appeared as sharply defined structures (Fig. 5) but in others they appeared somewhat fuzzy in appearance or occasionally as an electron-dense condensed mass between spherical particles (Fig. 4). Most of these bridges consisted of 2 parallel dense lines and their diameter was 13.2 ± 1.4 nm depending on the number of lines making a bridge. In case of fuzzy appearing bridges the diameter appeared larger but it was difficult to determine the number of dense lines making the bridge (Fig. 4). The bridges did not appear to be
Figure 3. Two stages of the budding-off process of BRSV from the cytoplasmic membrane of a Vero cell. Note the presence of symmetrically arranged dots (nucleocapsid) at the place of budding. Bar marker represents 200 nm.

Figure 4. Left: Spherical virus particles showing bridges (b) and dots (d). Note some bridges are attached to only one particle (arrow). Bar marker represents 200 nm; Right: Same field at higher magnification.
Figure 5. Upper: Spherical virus particles with and without internal structures. The bridges do not appear to be at a particular angle. Filamentous (f) form shows two parallel strands. Bar marker represents 200 nm.

Bottom: Same field at higher magnification showing detailed structures; b, bridges; bm, bilayer membrane; d, dots; f, filament. Bar marker represents 100 nm.
arranged at a particular angle. Some bridges which had only one end attached to a virus particle were also observed (Fig. 4).

Although the majority of virus particles appeared to mature at the cell surface, there were some indications of intracytoplasmic maturation. Mature spherical virus particles contained in a vesicle (Fig. 6) were observed. Inclusions containing thread-like material (nucleocapsid) were located in the cytoplasm. Empty membranous vacuoles (Fig. 7) of various sizes and forms were present in the matrix of the inclusion. A few spherical virus-like particles were observed within and at the periphery of the inclusions (Fig. 8).

**DISCUSSION**

The BRSV strain 375 infected Vero cells were primarily processed for confirming the presence of the virus in the infected cells. During this processing some of the interesting features of BRSV strain 375 were observed, which have not been reported previously. Thus it seemed appropriate to report our findings. These observations confirmed the reports of intracytoplasmic maturation and bridge formation between virus particles. In the present study the morphology of BRSV strain 375 was only observed in cultures showing extensive CPE. Our findings with respect to morphology are in general agreement with those of others [2, 7, 16]. However, the size of bridges as well as that of 'dots' of strain 375 were slightly larger than that of
Figure 6. An intracytoplasmic vesicle (v) showing four spherical (s) virus particles with and without internal structure. Bar marker represents 200 nm.

Figure 7. A large inclusion (I) showing membrane limited vacuoles (v) of variable size. Bar marker represents 400 nm.

Figure 8. An inclusion (I) showing spherical virus like particles (sv). Bar marker represents 500 nm.
strain A 51908. This difference in size could be due to cells or electron microscope used in this study. Filamentous and spherical particles without inner structure outnumbered the filaments as well as spherical particles having internal structure [14]. Probably only the latter are infectious; thus the consistently observed predominance of apparently incomplete virus might account for the generally low order of infectivity encountered with the RS viruses [7, 10, 11].

Well defined bridges between spherical particles were regularly observed. However they did not appear to be at a particular angle as reported by Belanger et al. [2]. Such bridges have not been reported for the Long strain of human respiratory syncytial virus or the NMK 7 strain of BRSV [2, 3, 7]. The available literature supports the view that these bridges are strain dependent [2] but their biological significance and origin are not currently known. The length of the bridges did not appear to exceed that of two spikes measured end to end, which gives rise to a possibility that these may be some form of viral spikes (condensed viral spikes), but the viral spikes in our study itself were not well defined. At least in one case the two bridges between two particles were not of the same size (Fig. 5), because of their position on the virus particles. So far no bridges or other regular structure connecting the filamentous forms have been reported. Belanger et al. [2] suggested that the high frequency of the presence of bridges in cross sections of virus particles indicate a longitudinal arrangement along filamentous forms. This was corroborated by observations of parallel and equidistant filamentous particles in longitudinal
sections; the space between filamentous forms appeared slightly electron-dense with occasional condensation of more electron-dense material. Most of the bridges observed by us are very discrete and do not appear to be the electron dense material in cross section. If the spherical particles are cross sections of filamentous forms [14] then some sort of connection between filamentous forms should be discernible. Another view could be that bridges are independent structures coded by the viral genome [2].

The BRSV strain 375 induced massive syncytia in Vero cells. Virus particles were seen budding from the plasma membrane and also lying outside or on the plasma membrane. However, spherical virus particles contained in vesicles were observed in the cytoplasm, which supports the findings of Moussa [13] regarding intracytoplasmic maturation of a BRSV strain. The difficulty experienced in achieving a high titer of BRSV in cell culture could also be partly due to intracytoplasmic maturation. We have observed that to produce a high titer of BRSV strain 375, 4-6 continuous passages are required in Vero cells (unpublished report). As such there is a possibility that with passage the overall population of Vero cells becomes conducive for viral replication while a minor subpopulation may have a restrictive effect leading to intracytoplasmic maturation. Abortive replication of influenza virus in nonpermissive HeLa cells has been reported. This restriction does not occur early in the virus growth cycle, but appears to be a defect in a late event resulting in large intracytoplasmic vesicles filled with budding particles [4].
Similarly, restriction on influenza virus can be imposed in permissive cells by treating the cells with concanavalin A [15]. Therefore intracytoplasmic maturation in Vero cells may be a host restricted factor rather than a character of the BRSV strains. Further studies are indicated, using different cell lines to minimize the host cell related variations to arrive at some final conclusion regarding the intracytoplasmic maturation of different strains of BRSV.

**LITERATURE CITED**


PRODUCTION, CHARACTERIZATION AND EPITOPE MAPPING OF MONOCLONAL ANTIBODIES GENERATED AGAINST N PROTEIN OF BOVINE RESPIRATORY SYNCYTIAL VIRUS

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Summary

A panel of monoclonal antibodies (MAbs), derived from the fusing of the primed BALB/C mice spleenocytes to SP2/O cells, were generated against the N protein of bovine respiratory syncytial virus (BRSV), strain 375. Different methods were used to immunize the mice for production of hybridomas and eventually MAbs. According to the class and subclass of Ig these MAbs can be grouped into four groups.

The MAbs were characterized by ELISA, a radioimmunoprecipitation assay, indirect immunofluorescence, and epitope mapping. To characterize these MAbs we used an ELISA competitive binding procedure based on the biotin-avidin system and enzyme-linked immunosorbent assay. Eight MAbs, two from each group, were used for epitope mapping. The competitive binding assay indicated competing and non
competing MAbs. The reaction pattern of the MAbs indicated 4 to 6 antigenic sites on the N protein.

Immune complexes consisting of radiolabeled BRSV and newly generated N protein MAbs, or radiolabeled BRSV and human respiratory syncytial virus N protein MAb were mixed and then electrophoresed together to monitor the electrophoretic mobility of the precipitated N proteins. The N proteins moved together indicating that they had the same molecular weight (43.4 Kd) and signifying that the generated MAbs were against the N protein of BRSV. This apparently is the first report of MAbs specific for N protein of BRSV.

Introduction

Economic losses due to respiratory disease in the United States cattle industry range from 250 to 750 million dollars annually.¹ Bovine respiratory syncytial virus (BRSV), an important pathogen of cattle, is considered to be a major contributor to the incidence of respiratory tract disease in nursing beef, feed lot and dairy calves.²³ Virus isolation and seroepizootiological studies have demonstrated that exposure of cattle to BRSV is widespread in many countries.⁴⁻⁵⁻⁶⁻⁷⁻⁸⁻⁹⁻¹⁰

BRSV is an enveloped, single stranded, negative sense RNA virus that has been classified with human respiratory syncytial virus (HRSV) as a Pneumovirus in the family Paramyxoviridae.¹¹ The viruses are antigenically related¹² and cause the
greatest morbidity in their respective species during the first six months of life when maternal immunity is still present. Reinfections are common and difficulties have been encountered in providing protection through live or inactivated vaccines against respiratory syncytial viruses (RSV). A major limitation of these vaccines is that maternally derived immunity to BRSV suppresses an active immune response to vaccination in calves.

No definitive studies appear to have been published regarding the specificity and duration of maternal immunity in calves. However, Westenbrink et al., in a very limited study, reported moderate levels of maternal antibody with specificity for the F and N proteins in three 2 to 3 wk old calves. Similarly, they observed in 6 to 7 month old calves, recovering from BRSV-associated disease, development of an antibody response, which was directed predominantly to the F and N proteins of the virus. Both F and N proteins of HRSV have been extensively used for inducing immunity in experimental animals. There appears to be only one report whereby immunoaffinity purified F protein of BRSV has been used for inducing immunity in calves. This report concluded that seronegative calves responded to the primary vaccination with F protein by producing neutralizing antibodies but subsequent vaccination did not boost the response, suggesting that already existing anti F antibody suppressed the immune response. Neither the production of monoclonal antibodies against N protein nor vaccination with N protein of BRSV has apparently been reported. Therefore the objective of the present study was to
generate monoclonal antibodies (MAbs) against N protein of BRSV strain 375, originally isolated from nasal secretions of a calf,\textsuperscript{21} and to characterize these by ELISA, indirect immunofluorescence, radioimmunoprecipitation assay (RIPA), and epitope mapping.

**Materials and Methods**

**Cells and viruses** - Vero cells (Veterinary Diagnostic Laboratory, College of Veterinary Medicine, ISU, CRL 1587 Vero 76 American Tissue Culture Collection) were maintained on Dulbecco's Modified Eagles's Medium [(DMEM), Sigma Chemical Company, St. Louis] supplemented with 10% betapropiolactone-treated fetal calf serum (Sigma) and 50 \( \mu \)g/ml of gentamicin (Schering Corporation, Kenilworth, NJ).

**Propagation of virus** - Vero cells grown in 175 cm\(^2\) tissue culture flasks (Costar Corporation, Cambridge, MA) were inoculated with BRSV strain 375 when the monolayer was subconfluent and incubated in a humidified chamber with 5% CO\(_2\) at 37\(^\circ\)C. A low titer of virus i.e., \(10^3\)/ml was obtained up to day 3 to 4 postinfection. Modification of the culture procedure resulted in a methodology that routinely yielded a titer of \(10^{6.5}\)/ml.

Cells were infected with virus (\(2\times10^9\)/ml) when the monolayer was about 80% complete. When viral cytopathic effect (CPE) was initially observed, usually 44-50h
post-infection, the cells were trypsinized and transferred with equal quantity of trypsinized unininfected Vero cells to a 225 cm² flask (Corning Incorporated, NY). On further incubation (24-48h) 90-100 % CPE was present and the cells were harvested to prepare antigen, for immunization of mice and western immunoblot assay (WIA).

Mouse myeloma SP2/0 cells (American Tissue Culture Collection CRL 1581) were grown in DMEM supplemented with 7.5% hybridoma serum (Sigma), 200 mM L-glutamine and 50 µg/ml gentamicin sulfate (Schering Corporation, Kenilworth, NJ) according to a procedure described by Van Deusen²² with some modifications. For growth of fusion products, first HMT medium (Sigma) and then HT medium (Sigma) was used. For preparation of these media DMEM was supplemented with 20% hybridoma serum, 20% conditioned medium, 2% HMT or HT (Sigma) along with gentamicin and L-glutamine as mentioned above.

**Generation of monoclonal antibodies**

**Immunization of mice:** Three preparations were used to prime 10-12 week old BALB/c mice for generation of MAbs against N protein of BRSV.

1. **Electrophoresed N-protein:** BRSV infected cells showing 90-100% CPE were centrifuged at 2000 g for ten min. The pellet was washed twice in DMEM (serum free). After a second washing, lysate buffer (150mM-NaCl, 1% sodium deoxycholate, 1% Triton x-100, 0.1% SDS, 10mM-Tris-HCl pH 7.4) was added at
10 x volume of the pellet and stirred overnight at 4°C. The preparation was centrifuged, the supernatant was collected and the protein content determined by the BCA protein assay (Bio-Rad Laboratories, Hercules, CA) and adjusted to 4 mg/ml with PBS (pH 7.2). This preparation was boiled at 100°C for five minutes with an equal volume of sample buffer consisting of 2% SDS, 50% glycerol and 0.05% bromophenol blue in 0.5M Tris buffer (pH 6.8). This viral antigen (100 ul) was run through 10% SDS-PAGE\textsuperscript{23} to resolve viral proteins. To locate the layout of the 43K Mr protein band gel strips (0.5 cm wide) were cut out from each side and stained with 0.1% Coomassie blue (Sigma). After destaining, both the cut out gel strips were placed in water to rehydrate and then placed back in their original position. The portion of unstained gel corresponding to the stained 43K Mr protein band was removed and stored at -70°C for further use. The band was freeze-thawed twice and ground to make a syringeable suspension in PBS. This suspension was inoculated intraperitoneally (i/p) into a group of mice. Three injections (Table 1) were given three weeks apart. Four days before fusion, the mice were injected i/p with 1x10\textsuperscript{7} BRSV infected cells.

2. **BRSV infected cells**: Another group of mice was inoculated i/p with whole BRSV infected cells (1x10\textsuperscript{7} cells/mice) as described by Klucas and Anderson.\textsuperscript{24} A booster dose was administered three weeks following primary immunization (Table 1). Four days before fusion, the mice were boosted with another dose of BRSV infected cells (1 x 10\textsuperscript{7} cells).
Table 1. Immunization of mice for generation of monoclonal antibodies.

<table>
<thead>
<tr>
<th>Type of immunogen</th>
<th>Weeks</th>
<th>Dose</th>
<th>Adjuvant</th>
</tr>
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<tbody>
<tr>
<td>Electrophoresed N protein</td>
<td>0</td>
<td>2-3 µg</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2-3 µg</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2-3 µg</td>
<td>No</td>
</tr>
<tr>
<td>N protein dissociated from immobilon membrane</td>
<td>0</td>
<td>6-8 µg</td>
<td>FCA</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6-8 µg</td>
<td>FIA</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6-8 µg</td>
<td>FIA</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>6-8 µg</td>
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<tr>
<td></td>
<td>12</td>
<td>6-8 µg</td>
<td>FIA</td>
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<tr>
<td>BRSV infected Vero cells</td>
<td>0</td>
<td>1x10^7 cells</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1x10^7 cells</td>
<td>No</td>
</tr>
</tbody>
</table>

FCA = Freund's complete adjuvant; FIA = Freund's incomplete adjuvant.

3. **N protein dissociated from immobilon membrane**: The viral antigen was prepared and electrophoresed by SDS-PAGE as previously described. The resolved proteins were transferred to an immobilon membrane (Millipore Corporation, Bedford, MA). Proteins were stained with 0.1% amido black (Sigma) and the 43K M_r protein band was excised and stored at 4°C. A group of mice was primed with N-protein dissociated from immobilon membrane according to a protocol described by Szewczyk and Summers. The dissociated N protein pellet was solubilized in PBS and incorporated into an equal volume of Freund's complete (Sigma) adjuvant to make water-in-oil emulsion and inoculated i/p into a group of mice. The boosting preparations were prepared similarly except that Freund's incomplete adjuvant...
(Sigma) was used. Five immunizations (Table 1) were carried out at three week intervals. Four days before fusion, the mice were boosted with BRSV infected cells as described previously.

**Fusion:** Four days after final injection, cell fusion was performed according to the procedure of Van Deusen. Hybridomas positive by ELISA and immunofluorescence were cloned by limiting dilution, rescreened and further characterized by radioimmunoprecipitation and epitope mapping.

**Ascites:** To generate ascites fluids containing monoclonal antibodies, mice were primed with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane) (Aldrich Chemical Company, Inc., Milwaukee, WI) i/p. Three weeks after priming, mice were inoculated i/p with $1 \times 10^6$ hybridoma cells, which were washed and resuspended in PBS. Ascites fluids were drawn 2-3 weeks post inoculation with a myelography needle (Becton-Dickinson, Rutherford, NJ), centrifuged, and stored at -70°C.

**Isotyping** - A mouse isotyping kit (Boehringer Mannheim Corporation, Indianapolis, IN) was used according to the procedure recommended by the manufacturer for determining the immunoglobulin class and subclass.

**Immunofluorescence assay** - Infected and uninfected Vero cells ($2 \times 10^4$ cells/well) were grown on teflon coated slides (ICN Biomedicals, Aurora, Ohio). When the CPE was well marked, the cells were fixed in 80% acetone at room temperature and
stored at -20°C. Hybridoma supernatant culture fluid was added to the wells, and bound antibody was detected by probing with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG or IgM [Kirkegaard & Perry Laboratories, Inc. (KPL), Gaithersburg, MD] diluted 1:40 in PBS. Processed slides were examined under ultraviolet light for fluorescence.

**Radioimmunoprecipitation assay** - BRSV-infected and Sham-infected Vero cells were radiolabeled according to the procedure of Bernstein and Hruska with some modifications. Vero cells were grown in a 75 cm² flask and infected with BRSV as described previously. Six hours after adding the uninfected cells to infected cells the medium was replaced with methionine-free MEM (Sigma), incubated at 37°C for one hour, and the medium was replaced with fresh methionine-free MEM containing 100 μCi/ml ^35^S-methionine (ICN Biomedicals, Inc., Irvine, CA). Twenty hours after the addition of ^35^S-methionine, when the CPE was over 80%, the cells were removed from the plastic surface with a scraper, and pelleted by centrifugation at 300 g for 15 min. The cell pellet was then disrupted with one ml of RIPA buffer, vortexed vigorously for one minute and placed on ice for three min. The cellular residue was removed by centrifuging at 300 g for 15 min. Mock-infected cell lysate was prepared by the same protocol.

Aliquots (50 μl) of labeled antigen were mixed with 50 μl of undiluted hybridoma supernatant. Antigen-antibody complexes were precipitated with a 30% suspension of
equal volumes of protein A and G Sepharose beads (Sigma), or anti-mouse IgM-
cross-linked beaded agarose (Sigma) according to the isotype of the hybridoma
supernatant. Similarly N monoclonal antibody (Courtesy Dr. Dan Speelman,
American Cyanamid Company, West Henrietta, NY) raised against HRSV was used
at 1:10 dilution for precipitating N protein from BRSV infected Vero cells. The
precipitated antigen-antibody complexes were subjected to SDS-PAGE on 10%
polyacrylamide gel. The gel was fixed in 7.5% acetic acid in deionized water,
incubated in autoradiography enhancer (Dupont, NEN Research Products, Boston,
MA), dried and exposed to X-OMAT film.

Electrophoretic mobility of N proteins immunoprecipitated by human and
bovine MAbs: Equal quantities of the immunoprecipitated complexes, prepared for
RIPA, containing HRSV MAb and BRSV MAb specific for N protein of the
respective viruses were mixed together. These complexes were subjected to SDS-
PAGE as already described for monitoring the electrophoretic mobility of the N
proteins precipitated both by human and bovine MAbs.

Enzyme-Linked Immunosorbent Assay

Preparation of antigens for ELISA: Cell and viral antigens were prepared
according to the method described by Bruckova et al. Both viral (+ag) and control
(-ag) antigens were diluted to 10 μg/ml in PBS containing 0.05% sodium azide
(PBSN). This preparation was used for coating the ELISA plates.
**ELISA procedure:** Immulon 4 microplates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 50 μl of PBSN (10 μg/ml ag) and incubated in a sealed zip-lock bag overnight at room temperature. Each plate contained alternate rows of +ag and -ag. Following the overnight incubation, the plates were stored at 4°C for no more than four months in a sealed container. Indirect ELISA as described by Coligan et al. was performed. Hybridoma culture supernatants diluted 1:5 were used as primary antibody. Peroxidase-labeled goat anti-mouse IgG (H+L) (KPL) (1:500) was used as secondary antibody. ABTS peroxidase substrate (KPL) was used for development of color and 1% SDS was used to stop the reaction. Absorbance values were determined with a kinetic microplate reader (Molecular Devices Corporation, Menlo Park, CA) with a test filter wavelength of 405 nm.

**Epitope mapping:** Eight monoclonal antibodies, two from each group, were processed for epitope mapping according to the procedures described previously with some modification as mentioned below.

The protein concentration of the precipitated antibodies was adjusted to 1.5 mg/ml with 0.1 M Na HCO₃ buffer. For the purpose of biotinylation 1.0 mg of biotinamidocaproate N-hydroxysuccinimide ester (Sigma) was dissolved in 1.0 ml of dimethyl sulfoxide (Sigma). One hundred μl of it was immediately added /ml of the MAb and allowed to react for 2h at room temperature with periodic mixing.

For competitive ELISA both the labeled and unlabeled MAbs were diluted 1:2 with glycerol. The ELISA procedure was essentially the same as already described
with the following changes. Plates were coated with 100 μl of +ag (30 μg/ml) in coating buffer (0.01M sodium borate, pH 9.2) and incubated at 4°C for 18h. The optimum dilution of the biotinylated MAbs was determined by diluting 1:10 to 1:10,240 in blocking buffer (0.01 M sodium borate with 2% BSA) and analyzed with no competing MAbs. Since a 1:100 dilution at the rate of 100 μl/well gave a consistent OD value of one, the biotinylated MAbs were used at this dilution in the competitive ELISA. Two procedures were used for monitoring the competition. In the first procedure, the unlabeled MAbs (75 μl/well) in four different concentrations (1:25 to 1:100) were incubated at room temperature for 18h. Without aspirating the wells, 25 μl of biotinylated MAbs were added and the plate incubated at 37°C for two hours.33 In the second procedure, the biotinylated and four different concentrations (1:25 to 1:100) of unlabeled MAbs were incubated simultaneously (50 ul each/well) and the plate incubated at 37°C for two hours.34 After washing the plates, 100 ul of streptavidin peroxidase (KPL) diluted to 1:4000 in PBS with 1% BSA was added to each well and the plates incubated at 37°C for one hour. The plates were washed and further processed as described earlier. The percentage of competition was calculated according to the formula: (1-A/A_max) 100, where A is the mean of the specific absorbance of wells with the competing MAbs, and A_max is the mean of the specific absorbance for wells with diluent in place of competing MAbs. The competition was considered significant when the inhibition was 50% or more.
Results

*Immune response* - A panel of hybridoma cell lines secreting MAbs specific for N protein of BRSV were generated employing three different procedures. The MAbs generated in this study could be grouped into four groups (Table 2) according to Ig class and subclass.

<table>
<thead>
<tr>
<th>Group</th>
<th>Antibody class and subclass</th>
<th>Designation of MAbs</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>IgM, $\lambda$</td>
<td>65-12 and 65-16</td>
</tr>
<tr>
<td>C</td>
<td>IgG$_1$, $\kappa$</td>
<td>25-0, 25-2, 25-3 and 25-7</td>
</tr>
<tr>
<td>D</td>
<td>IgG$_{2\alpha}$, $\kappa$ &amp; $\lambda$</td>
<td>38-6, 38-7, 38-8 and 38-9</td>
</tr>
</tbody>
</table>

Group A and group B MAbs were generated from the same fusion, originating from two wells 65 and 61. For generation of these MAbs BALB/c mice were primed with electrophoresed N protein contained in polyacrylamide gel strips. The hybridomas of these wells were cloned, screened and consequently 11 subclones were raised. Most of these subclones produced IgM, $\kappa$ and only two produced IgM, $\lambda$ antibodies. Group C and group D MAbs originated from single well 25 and 38 respectively from separate fusions. These MAbs were generated from BALB/c mice primed with BRSV infected cells and N protein disassociated from immobilon membrane respectively.
Characterization of MAbs

ELISA: Primary and cloned hybridomas giving an OD value of 0.9 or greater with viral antigen and 0.15 or less with cell antigen were selected for growing for collection of supernatant for further studies.

Immunofluorescence: The immunofluorescence pattern we observed with these MAbs revealed localized nucleoprotein in the cytoplasm in early infection cycle (Fig. 1). Neither annular fluorescence nor peripheral filamentous extensions protruding from the cell surface, consistent with specificity for a protein expressed on the cell surface, were observed.

Radioimmunoprecipitation assay: The hybridoma supernatants were used to immunoprecipitate polypeptides from virus-infected and uninfected Vero cells, labeled with $^{35}$S-methionine. The immunoprecipitates were analyzed on 10% polyacrylamide gel. The pattern of RIPA reactions indicated that all the MAbs precipitated a 43 Kd protein from viral infected cells (Fig. 2, 3, 4). None of the MAbs precipitated proteins from uninfected cells, suggesting that these MAbs were specific for the 43 Kd viral protein, which is the N protein. This specificity was confirmed by comparison with immunoprecipitation pattern formed by a reference MAb (MAb against N protein of Long strain of HRSV). The reference MAb precipitated a protein of identical size (Fig. 2) and, when complexes formed by test and reference MAbs were mixed, and subjected to electrophoresis a single band of precipitate was observed. Both the N proteins precipitated by human and bovine MAbs migrated
Figure 1. Intracytoplasmic fluorescence in BRSV infected Vero cells produced by N protein monoclonal antibody. The cells were fixed in acetone and air dried. Wells were incubated with hybridoma supernatant followed by a 1:40 dilution of fluorescein-labeled anti-mouse IgG antibody.
Figure 2. Radioimmunoprecipitation of $^{35}$S-methionine-labeled BRSV strain 35 infected and uninfected Vero cells with D$_{14}$ (MAb raised against N protein of HRSV) and 61-1 (MAB raised against N protein of BRSV). Hybridoma supernatant of 61-1 MAb and ascites fluid of MAb D$_{14}$ diluted 1:10 in PBS was used to immunoprecipitate polypeptides from BRSV infected and uninfected Vero cells. $^{14}$C-methylated proteins as molecular weight markers. Lane 1 and 3 = Uninfected Vero cells precipitated with MAb D$_{14}$ and 61-1 respectively. Lane 2 and 4 = Infected Vero cells precipitated with D$_{14}$ and 61-1 respectively. Antibody-antigen complexes were collected with Protein A and G coated Sepharose beads and antimouse IgM-agarose respectively. The complexes were subjected to SDS-PAGE on 10% polyacrylamide gel. The gel was fixed in acetic acid, dried and exposed to X-OMAT film.

Figure 3. Radioimmunoprecipitation of $^{35}$S-methionine-labeled infected and uninfected Vero cells with three MAbs (65-12, 65-16 and 38-5) directed against N protein of BRSV. Lane 1 = $^{14}$C-methylated proteins as molecular weight markers. Lane 2, 4 and 6 = Uninfected Vero cells precipitated with N protein MAbs of BRSV. Lane 3, 5 and 7 = BRSV infected Vero cells precipitated with N protein MAbs of BRSV showing 43 Kd precipitated protein. Hybridoma supernatants were used to immunoprecipitate polypeptides from BRSV infected and uninfected Vero cells. Antibody-antigen complexes were collected with either Protein A and G coated Sepharose beads or antimouse IgM-agarose according to the isotype of the MAb. Further processing was as mentioned above. Since 38-5 indicated some reactivity with viral protein other than N protein, it was discarded.
Figure 4. Radioimmunoprecipitation of $^{35}$S-methionine-labeled infected Vero cells with seven MAbs directed against N protein of BRSV and one MAb ($D_{14}$) directed against N protein of HRSV. Lane 1 and 10= $^{14}$C-methylated proteins as markers. The markers were added on both sides to measure exactly the molecular weight of N protein by image analysis which, was found to be 43.4 Kd. Lane 2=Mab 38-6. Lane 3=MAb 25-2. Lane 4=MAb 65-12. Lane 5=MAb 61-1. Lane 6=Mab $D_{14}$. Lane 7=MAb 61-19. Lane 8=Mab 61-13. Lane 9=MAb 61-7. Hybridoma supernatants and ascites fluid ($D_{14}$) were used to immunoprecipitate polypeptides from BRSV infected Vero cells. The complexes were subjected to SDS-PAGE on 10% polyacrylamide gel. The gel was fixed in acetic acid, dried and exposed to X-OMAT film.

Figure 5. Radioimmunoprecipitated complexes (RIPC) precipitated by N protein MAbs of BRSV and N protein MAb of HRSV were mixed and subjected to SDS-PAGE on 10% polyacrylamide gel. The gel was fixed in 7.5% acetic acid, dried and exposed to X-OMAT film. $^{14}$C-methylated proteins as markers. Lane 1=RIPC precipitated by $D_{14}$. Lane 2=RIPC precipitated with $D_{14}$ and 38-7. Lane 3=RIPC precipitated with 38-6. Lane 4=RIPC precipitated with $D_{14}$ and 65-16. Lane 5= RIPC precipitated with 65-16. Lane 6=RIPC precipitated with $D_{14}$ and 61-1. Lane 7=RIPC precipitated with 61-1. Lane 8=RIPC with $D_{14}$ and 25-2.
together and did not separate (Fig. 5) on electrophoresis, which further confirmed that the generated MAbs were specific for the N protein of BRSV.

**Epitope mapping:** The two procedures which were used for epitope mapping gave somewhat different results. The staggered incubation procedure identified 4 antigenic sites (A,B,C,and D) on the N protein (Fig. 6). MAbs 25-0, 25-3, 38-7, and 38-9 competed over 50% with all other MAbs, signifying that these MAbs bind to an identical epitope (Antigenic site A). Although 61-1 and 61-7 appear to bind a similar epitope (Antigenic site B) but these two MAbs exhibited a lower competition with 25-0 and 25-3. This may be an overlapping epitope or a distinct epitope. MAbs 65-12 and 65-16 compete over 50% with 50% and 62% of all the MAbs respectively but their reactivity with the remaining MAbs is markedly different, suggesting these two interact with independent antigenic sites C and D respectively (Table 3).

The simultaneous incubation procedure indicated 6 epitopes (A',B',C',D',E and F) (Fig. 7) rather than 4 epitopes. In particular, antigenic site D' appears to be entirely different from the other antigenic sites. MAb 38-9 exhibited far less competition with all other MAbs (Table 4). This could be due to the epitope itself, or the binding of MAbs may have altered the site to such an extent that other MAbs could not bind, or the competition was low because of some steric hindrance. Antigenic site A apparently appears to be similar to the antigenic site A'. Antigenic site E appears to a large extent to be similar to site B. Antigenic site C' is similar to
Figure 6. Competitive binding of anti-N MAbs. Unlabeled MAbs were incubated for 18h at room temperature in microtiter plates, coated with BRSV antigen and then biotinylated MAbs were added. After 2h incubation at 37°C, bound antibody was detected using peroxidase labeled streptavidin and ABTS enzyme substrate. The results are expressed as percent competitive inhibition.
Table 3. Competitive ELISA between non-biotinylated and biotinylated MAbs

<table>
<thead>
<tr>
<th>Biotinylated MAb</th>
<th>Percent competition with the following competing MAbs</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>25-0</td>
</tr>
<tr>
<td>25-0</td>
<td>68.7</td>
</tr>
<tr>
<td>25-3</td>
<td>70.3</td>
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<td>38-7</td>
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<td>19.0</td>
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<td>65-16</td>
<td>27.5</td>
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</table>

Competitive binding of anti-N MAbs. Unlabeled MAbs were incubated for 18h at room temperature in microtiter plates, coated with BRSV antigen and then biotinylated MAbs were added. After 2h incubation at 37°C, bound antibody was detected using peroxidase labeled streptavidin and ABTS enzyme substrate. The results are expressed as percent competitive inhibition.

Table 4. Competitive ELISA between non-biotinylated and biotinylated MAbs

<table>
<thead>
<tr>
<th>Biotinylated MAb</th>
<th>Percent competition with the following competing MAbs</th>
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<tr>
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<td>65-12</td>
<td>53.6</td>
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<td>65-16</td>
<td>44.1</td>
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</table>

Competitive binding of anti-N MAbs. Biotinylated MAbs and unlabeled MAbs were put together in microtiter plates, coated with BRSV. After 2h at 37°C, bound antibody was detected using peroxidase-labeled streptavidin and ABTS enzyme substrate. The results are expressed as percent competitive inhibition.
Figure 7. Competitive ELISA of anti-N MAbs. Biotinylated MAbs and unlabeled MAbs were put together in microtiter plates, coated with BRSV. After 2h at 37°C, bound antibody was detected using peroxidase-labeled streptavidin and ABTS enzyme substrate. The results are expressed as percent competitive inhibition.
A and A' but it shows a lower competition with MAb 25-0. The remaining two antigenic sites, namely B' and F, appear to be markedly distinct from other sites.

Discussion

A panel of MAbs specific for N protein of BRSV were generated by priming BALB/c mice with three different viral preparations. Each preparation induced a different immune response, which is in agreement with the recognized principle that the type of adjuvant and manner of preparation of antigen can effect the immune response. These MAbs can be divided into four different groups according to their Ig class and subclass.

The immunofluorescence pattern we observed with these MAbs revealed localized nucleoprotein in the intracellular location early in the infection cycle. Neither annular fluorescence nor peripheral filamentous extensions protruding from the cell surface was observed as has been reported with MAb raised against N protein of HRSV.

Immunoprecipitation of radiolabeled proteins is a commonly used procedure for identifying the protein specificity of MAbs. Radioimmunoprecipitation assays conducted during this study further confirmed that the generated MAbs were against the N protein because the molecular weight of the precipitated protein was found to be 43 Kd. In addition, the MAbs did not precipitate with cell protein. The
molecular weight of N protein of HRSV and BRSV has been reported to be 41 Kd to 44 Kd and 43 Kd respectively.\textsuperscript{27,37,38,39}

To characterize the interrelationship of the MAbs against the N protein of BRSV and its epitopes, we used two procedures of a competitive binding assay\textsuperscript{35,34} based on the biotin avidin system and ELISA. This assay measured the extent of competition between competing and non competing MAbs. Failure of unlabeled antibody to reduce the binding of labeled antibody, provided evidence that the antibodies bind to distinct sites on the antigen. However, this could also be due to the fact that the labeled antibody binds with higher avidity than the unlabeled competitor to an overlapping epitope.\textsuperscript{40}

Both one way and two way competition was observed between some of the MAbs in both the procedures. Two way blocking between two MAbs suggests, but does not prove, that they react with the same or an overlapping epitope. Conformational changes as well as steric hindrance can result in blocking between two MAbs which otherwise would not have had a blocking effect. Similarly, the lack of blocking between two MAbs suggests, but does not prove, that these MAbs react with distinct antigenic sites or epitopes.\textsuperscript{41} The lack of blocking could be one-way or two-way. The former could be due to differences in avidity and the latter could be an artifact of the preparations of the viral antigen used as the immunogen.\textsuperscript{41} Three procedures were used in this study for the preparation of viral antigen for immunizing. There is a good possibility that two or more populations of the same protein could be
produced having conformational or altered epitope resulting in differences in their reactivity with different MAbs.41

Keeping in view some of the above referred limitations we used two procedures whereby the labeled and unlabeled MAbs were applied together or one after the other sequentially. The application of labeled and unlabeled MAbs was also reversed. Our competitive assays presumptively identified 4 to 6 antigenic sites on the N protein of BRSV. Some of them were similar and others appeared quite distinct. One example would be antigenic site D' (Fig. 5). It appears that binding of unlabeled MAbs altered the site to such an extent that the labeled MAb is far less competitive. In the other procedure, this MAb has over 50% competition with all the other MAbs, indicating that most probably the procedure itself has had a marked effect on the competition between MAbs. Based on the competitive binding ELISA test, 15 epitopes have been reported on the N protein of HRSV.

All the data indicate that the MAbs generated in this study are specific for the N protein of BRSV and that there are at least 4-6 antibody-binding sites on the N protein.

Literature Cited


IMMUNIZATION AGAINST BOVINE RESPIRATORY SYNCYTIAL VIRUS WITH SOLID MATRIX-ANTIBODY-ANTIGEN COMPLEXES CONTAINING N PROTEIN OF THE VIRUS

A manuscript to be submitted to American Journal of Veterinary Research

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Summary

In the present study two monoclonal antibodies, specific for N protein of BRSV, 65-12 (IgM) and 38-6 (IgG₂), were used to make antibody-antigen complexes by incubating these with BRSV infected cell lysate. The antibody-antigen complexes were adsorbed to a solid matrix by incubating these complexes with antimouse IgM-agarose and Protein A-agrose beads according to the isotype of the MAbs. The SMAA complexes so formed were washed five times with PBS, suspended in an equal quantity of aluminum hydroxide gel and used for immunization of two groups of lambs. Immune responses were monitored by the lymphocyte transforming assay (LTA), western immunoblot assay (WIA) and serum neutralization (SN) assay.

In vaccinated groups, the maximum immune response in terms of SI* (stimulation index), determined by LTA, was observed on day 11 postvaccination. However, the response of animals in group B, vaccinated with SMAA complexes containing MAb (IgM), was significantly higher than that of the group C, vaccinated with SMAA
complexes containing MAb (IgG₂κ). Immune responses were also determined by the western immunoblot assay. By 11-days postvaccination four animals from group B exhibited a marked response (band) to the N protein and three animals from group C showed a very faint band at the same position. WIA did not detect F or G proteins.

Challenging 21 days postvaccination with heat inactivated BRSV resulted in a dramatic neutralizing antibody responses in lambs primed with SMAA complexes. Appreciable titers were present in the sera five days following immunization and titers peaked at 14 days. In contrast, antibody titers were barely detectable in control animals at five days and relatively low titers were present even at 21 days. This clearly indicated enhanced responses to neutralizing epitopes associated with F and G proteins and this was confirmed by WIA conducted on these sera. We conclude that immunization with N protein primed these animals for a response to other structural proteins of the virus.

**Introduction**

In spite of all efforts and modern scientific developments, bovine respiratory disease (BRD) continues to be an enigma for the cattle industry of many countries including the United States of America. It has been estimated that 40-80% of all diseases of cattle involve the respiratory system.¹ In the United States alone it causes a loss of 250-750 million dollars annually.² Baker³ cited a 1977 Livestock
Conservation Institute estimate of one billion dollars loss due to BRD per year in North America.

Bovine respiratory syncytial virus (BRSV) is one of the most frequently occurring and virulent etiological agents of respiratory tract disease in calves. Virus isolation and seroepizootiologic surveys have indicated that exposure of cattle to BRSV is widespread in many countries.

Human as well as bovine respiratory syncytial virus belong to the genus Pneumovirus of the family Paramyxoviridae. Both viruses are antigenically related and cause the greatest morbidity in their respective species during the first six months of life when maternal immunity is still present. Reinfections are common and difficulties have been encountered in providing protection through live or inactivated vaccines against respiratory syncytial viruses. A major limitation of these vaccines is that maternally derived immunity to BRSV suppresses an active immune response to vaccination in calves. The basis for this suppression is not well understood. It may result from the blocking effect of the specific antiviral antibodies. Nelson et al. reported that seronegative calves responded to the primary vaccination with immunopurified F protein by producing antibodies. Subsequent vaccination did not boost the response, suggesting that already existing anti F antibody suppressed the immune response.

The genome of the respiratory syncytial viruses codes for ten proteins, including two nonstructural (1B and 1C) and eight structural proteins. The major
glycoprotein (G), fusion protein (F), 1A protein and 22Kd proteins are components of the viral envelope. The nucleocapsid contains the nucleocapsid protein (N), the phosphoprotein (P), and the large protein (L). The matrix protein (M) forms a structural layer between the envelope and the nucleocapsid. Antibodies to all the structural proteins develop in convalescent calves (6 to 7 month old), but the predominant response is directed against the F and N proteins. Similarly, in a limited study, 2 to 3 week old calves have been reported to have moderate levels of maternal antibodies to the F and N proteins against BRSV.

A number of workers have analyzed the antibody response to N protein of HRSV in experimental animals with variable results. King et al. reported that N protein expressed in vaccinia virus afforded a significant degree of protection to challenge in BALB/c mice. Although vaccination with the recombinant expressing N protein resulted in a significant reduction in titer in the lung of the mice following live virus challenge, the protection was not as solid as provided by G and F proteins expressed and used similarly. Peretz et al. have reported that G and F but not N protein induced protection in BALB/c mice. Immunity induced by G or F proteins lasts longer than that induced by N protein. These observations suggest that N protein is not as immunogenic as the G and F proteins and thus maternal antibody to the N protein may drop below detectable levels sooner than antibody to the F protein. Our own preliminary studies (unpublished data) in which the specificity and duration of
maternal antibody was studied suggested that antibody to the N protein drops below detectable levels before antibody to the F protein.

The N protein probably plays a role in RSV-specific cytolytic T-cell responses, because N protein served as a target for RSV-specific Tc cells in both mouse and human studies. It is therefore reasonable to assume that the N protein of BRSV may also serve as a target for bovine Tc cells.

The role of nucleocapsid protein as a protective antigen has also been described for the morphologically similar influenza virus. Over a series of experiments 75% mice were protected and the protective response induced by the nucleocapsid protein, like that induced by N protein of RSV, appeared to be cell mediated in nature.

Immunization of mice with solid matrix-antibody-antigen complexes (SMAA), containing either internal or external structural proteins from the same virus (Simian virus 5) or from different viruses (herpes simplex virus, influenza virus, measles virus and simian virus), have been reported to induce vigorous humoral and cell mediated immune responses. The N protein of BRSV has not been used as immunogen in any form individually. Through this study it was envisaged to use SMAA complexes containing N protein of BRSV to investigate its immunogenicity in lambs and to determine the carrier effect on subsequent challenge with heat inactivated virus. Several workers have reported successful vaccination with proteins from virus incorporated into SMAA complexes.
Materials and Methods

**Cells and viruses** - Vero cells (Veterinary Diagnostic Laboratory, College of Veterinary Medicine, ISU., CRL 1587 Vero 76 from American Tissue Culture Collection) were maintained on Dulbecco's Modified Eagles's Medium [(DMEM), Sigma Chemical Company, St. Louis] supplemented with 10% betapropiolactone-treated fetal calf serum (Sigma) and 50 μg/ml of gentamicin (Schering Corporation, Kenilworth, NJ).

**Propagation of virus** - Vero cells grown in 175 cm² tissue culture flasks (Costar Corporation, Cambridge, MA) were inoculated with BRSV strain 375 when the monolayer was subconfluent and incubated in a humidified chamber with 5% CO₂ at 37°C. A low titer of virus i.e., 10⁷/ml was obtained up to day 3 to 4 postinfection. Modification of the culture procedure resulted in a methodology that routinely yielded a titer of 10⁶.5/ml.

Cells were infected with virus (2x10⁴/ml) when the monolayer was about 80% complete. When viral cytopathic effect (CPE) was initially observed, usually 44-50h postinfection, the cells were trypsinized and transferred with equal quantity of trypsinized uninfected Vero cells to a 225 cm² flask (Corning Incorporated, Corning, NY). On further incubation (24-48h post infection), 90-100 % CPE was present and the cells were harvested to prepare vaccine, for immunization of lambs and antigen for lymphocyte blastogenesis and western immunoblot assay (WIA).
**Preparation of SMAA complexes**

**Preparation of cell lysate:** BRSV infected Vero cells showing 90-100% CPE were centrifuged at 2000 g for ten min. The pellet was washed twice in DMEM (serum free). After the second washing, lysate buffer (150mM-NaCl, 1% sodium deoxycholate, 1% Triton-x-100, 0.1% SDS, 10mM-Tris-HCl pH 7.4) was added at a 10 x volume of the pellet and stirred overnight at 4°C. The preparation was again centrifuged as before and the supernatant fluid was collected. The cell lysate was sonicated for 30 seconds at 6.5K, 50% cycle on ice, centrifuged and supernatant collected.

**Preparation of antigen-antibody complexes:** Tissue culture supernatants from two monoclonal antibodies (MAb) 65-12 (IgM) and 38-6 (IgG2a), specific for N protein of BRSV, were used for preparation of antigen-antibody complexes. Five ml each of the MAb supernatant was incubated overnight at 4°C with 12.5 ml of cell lysate on a rocker platform.

**Preparation of washed SMAA complexes:** The antigen-antibody complexes consisting of cell lysate and MAb 65-12 or 38-6 were adsorbed onto a solid matrix by addition of six ml of anti-mouse IgM-agarose (Sigma) and 1.25 ml protein A-agrose beads (Sigma) respectively and incubated for another 4h at 4°C. The SMAA complexes so formed were washed five times with phosphate buffered saline (PBS, pH 7.2) by centrifugation at 2000 rpm for ten min and adjusted to 10 ml by addition of PBS. The SMAA complexes were suspended in an equal quantity (v/v) of one
percent aluminum hydroxide gel (Intergen Company, Purchase, NY) for immunization of lambs.

**Experimental design**

**Immunization:** Fifteen lambs, five to six months of age and seronegative to BRSV, were randomly divided into three equal groups. One group served as a control and the other two groups were immunized with SMAA complexes.

**Group A** served as a control. All animals were injected with one ml of 0.5% aluminum hydroxide gel subcutaneously at a site on each side of the neck.

**Group B** animals were injected subcutaneously with two ml of SMAA complexes consisting of N protein of BRSV, MAb (IgM), antimouse IgM-agrose and aluminum hydroxide gel at a site on each side of the neck.

**Group C** was injected subcutaneously with two ml of SMAA complexes consisting of N protein of BRSV, MAb (IgG2a), Protein A-agrose beads and aluminum hydroxide gel at a site on each side of the neck.

Blood samples (30 ml) were collected prior to immunization and on days 7, 11, 15 and 21 postvaccination for lymphocyte blastogenesis, western immunoblot assay (WIA) and serum neutralization (SN) assays. For serum, blood was allowed to clot overnight and then centrifuged for 30 min at 2000 rpm after which the serum was collected, aliquoted and stored at -20°C.
Challenge: BRSV-infected Vero cell suspension (300 ml, titer \(10^{5.8}/\text{ml}\)) was inactivated at 56°C for 30 min. This virus preparation was lyophilized and reconstituted in 30 ml PBS and dialyzed against two changes of PBS for 36h at 4°C. All animals were injected s/c with 2 ml of the virus preparation three weeks after primary vaccination.

All animals were bled and serum harvested on days 5, 8, 11, 14 and 21 after challenge with inactivated virus. The immune response was monitored by WIA and SN assay.

*Lymphocyte blast transforming assay* - The lymphocyte transforming assay was conducted by measuring the incorporation of \(^3\text{H}-\text{thymidine into DNA by cultured lymphocytes. For this purpose the peripheral lymphocytes were harvested from the anticoagulated venous blood according to a modified procedure of Boyum.}^30\) Histopaque 1077 (Sigma) was diluted with 13% Hank's balanced salt solution (HBSS, without calcium and magnesium, Gibco Laboratories, Grand Island, NY). Briefly seven ml of diluted blood were carefully layered onto four ml of prewarmed Histopaque and centrifuged at 250 g for 30 min. The lymphocyte-rich interface was aspirated with a pipet and transferred to a tube containing a 2X volume of HBSS and again centrifuged at 150 g for 15 min. The supernatant was decanted and the pellet resuspended in four ml of HBSS. Cell concentration was determined with a Coulter Counter (Coulter Electronics Inc., Hialeah, FL). Trypan blue was used for
determining the viability of the cells and only those preparations were processed for blastogenic assay which had more than 80% viable cells. Each cell sample was diluted to a concentration of 200,000 cells/200 μl with diluting media containing 89% RPMI (Gibco), one percent antibiotic-antimycotic 100 X (Penicillin-G, Streptomycin sulphate and Amphotericin-B, Sigma) and 15% fetal calf serum (Intergen). An aliquot of 200 μl of diluted cell suspension was dispensed in flat bottom 96 well cell culture plates. Three wells were used for each mitogen and antigen per animal and three wells were left as untreated controls for each animal. The mitogens were used at a dose rate of 25 μl mitogen (6.25 μg)/well and the same quantity of the medium was added to cells of the control wells. BRSV antigen prepared according to a protocol of Bruckova et al., was used at a protein concentration of 60 μg/well. The plates were incubated for 72h at 37°C in a CO2 incubator. The plates were labeled with ³H-thymidine (0.75 μCi/well; ICN, Irvine, CA) and incubated for another 18h. The cells were harvested on fiberglass filters with a PHD cell harvester (Cambridge, Watertown, MA). Filter paper discs were transferred to scintillation vials containing Scintiverse BD (Fisher Scientific, Pittsburgh, PA) and the radioactivity determined with a scintillation counter (Packard Instrument Co., Downers Grove, IL). The stimulation index (SI) was calculated by dividing the mean value of stimulated lymphocyte cell cultures by the mean value of the unstimulated lymphocyte cell cultures from a single animal. The results were analyzed by analysis of variance with a 95% confidence level.
Western Immunoblot Assay

Preparation of antigens: BRSV-infected cells showing 90-100% CPE were centrifuged at 2000 g for ten min. The pellet was washed twice in DMEM (serum free). After a second washing, lysate buffer (150mM-NaCl, 1% sodium deoxycholate, 1% Triton x-100, 0.1% SDS, 10mM-Tris-HCl pH 7.4) was added at a 10 x volume of the pellet and stirred overnight at 4°C. The preparation was centrifuged, the supernatant was collected and the protein content determined by the BCA protein assay (Bio-Rad Laboratories, Hercules, CA) and adjusted to 4 mg/ml with PBS. This preparation was boiled at 100°C for five minutes with an equal volume of sample buffer consisting of 2% SDS, 50% glycerol and 0.05% bromophenol blue in 0.5M Tris buffer (pH 6.8). This viral antigen (100 ul) was run through 10% SDS-PAGE\(^3\) to resolve viral proteins.

SDS-PAGE analysis: A vertical slab mini-gel apparatus (Bio-Rad) was used for electrophoresis as directed by the manufacturer. Prestained SDS-PAGE standards (Bio-Rad) ranging from 18.5-106 Kd were used as molecular mass standards. For immunoblotting the electrophoresed proteins along with molecular weight markers were electrophoretically transferred to a 0.45 μ nitrocellulose membrane (Bio-Rad). Transfer was carried out at 4°C for 90 min at 100 volts in transfer buffer consisting of 25mM Tris, 192mM glycine, and 20% v/v methanol (pH 8.3).

Immunoblot assay procedure: The nitrocellulose membrane having separated proteins was blocked overnight at 4°C in TBS (500 mM NaCl, 20mM Tris, pH 7.5)
containing 1% gelatin (Difco Laboratories, Detroit, Michigan). The membrane was washed for three five minute cycles in TTBS (TBS containing 0.05% Tween 20) on a rocker platform. The membrane was incubated with test lambs serum or MAb specific for N protein\textsuperscript{33} diluted 1:100 in PBS (pH 7.2) for one hour at room temperature on a rocker platform. Miniblotters 16 and 28 (Immunetics, Cambridge, MA) were used for incubating the serum and MAb. The membrane was again washed and incubated in peroxidase-labeled anti species IgG (L+H) antibody (KPL) for one hour at room temperature on a rocker platform. The membrane was washed and bound IgG was visualized by immersing the membrane in three component TMB membrane peroxidase substrate (KPL). The color reaction was allowed to develop for 2-3 minutes and then stopped by three brief washes in deionized water. The molecular weights were determined by comparison with the protein standards\textsuperscript{34} and image analysis.

\textit{Serum virus neutralization assay} - Serum-virus neutralization (SN) was performed on lambs serum samples as described previously.\textsuperscript{35} Briefly, all sera were heat inactivated at 56°C for 30 min. Two-fold dilutions of sera were made in MEM in microtiter plates (Costar) in a way that each well contained 50 ul of serum dilution. Virus was diluted to 100 TCID\textsubscript{50}/50 ul, in MEM and added to all wells at the rate of 50 ul/well, and the mixture incubated at 37°C for one hour. A suspension of approximately 2x10\textsuperscript{4} BT cells/well was added in 150 ul of growth media and the
plates incubated in a CO₂ incubator at 37°C for one week. The serum-virus neutralization titer was the highest dilution of serum in which 50% of the cells in a well were protected from the viral CPE. The results were analyzed by analysis of variance with a 95% confidence level.

Results.

Lymphocyte transformation responses - All the results are the mean of triplicate cultures. The peripheral blood lymphocyte blastogenic response in the form of stimulation index (SI), as measured by the uptake of ^H-thymidine by DNA of lymphocytes of the lambs in all groups, are presented in Table 1. The mean SI of BRSV antigen-stimulated cultures significantly increased in both immunized groups (Table 1) as compared to the control group. However, the response in group B, vaccinated with SMAA complexes containing MAb (IgM), was significantly higher than group C, vaccinated with SMAA complexes containing MAb (IgG₂a) (Fig. 1). The SI in group B continuously increased from 1.26 ± 0.66 before vaccination to 2.99 ± 1.89 at 7 days and 3.20 ± 1.60 at 11 days postvaccination. Even at 21 days postvaccination the SI of group B was apparently higher than the group C and the control group, but statistically it was insignificant. The maximum response in both the vaccinated groups occurred at day-11 postvaccination. However, in group C the response was of low magnitude, declined by 15 days postvaccination and was essentially equivalent to the control group on day-21 postvaccination.
Table 1. Antigen induced stimulation indexes (mean±SE) of different groups of lambs

<table>
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<tr>
<th>Group*</th>
<th>Day-0</th>
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<th>Day-11</th>
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<td>Ag</td>
<td>Ag</td>
<td>Ag</td>
<td>Ag</td>
<td>Ag</td>
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<tr>
<td>A</td>
<td>1.11* ± 0.15</td>
<td>1.23b ± 0.29</td>
<td>1.21b ± 0.17</td>
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<td>2.99a ± 0.84</td>
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<td>LSD</td>
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<td>1.60</td>
<td>1.67</td>
<td>1.09</td>
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</table>

*Each group had five lambs.

LSD = least significant difference (P=0.05).

Means with same superscript not significantly different based on the LSD.

Peripheral blood lymphocytes were cultured with BRSV antigen at a protein concentration rate of 60 µg/well, before vaccination as well as after vaccination, as indicated above. After 96 hours the cultures were labeled with ³H-thymidine and harvested at 18 hours later. Stimulation indexes (SI') are average of three wells. The SI' were calculated by dividing the stimulated mean value of lymphocyte cell cultures by the mean value of the unstimulated lymphocyte cell cultures of the respective wells.
Figure 1. Peripheral blood lymphocytes were cultured with BRSV antigen at a protein concentration rate of 60 μg/well, before vaccination as well as after vaccination. After 96 hours the cultures were labeled with ³H-thymidine and harvested at 18 hours later. Stimulation indexes (SI) are average of three wells. The SI were calculated by dividing the stimulated mean value of lymphocyte cell cultures by the mean value of the unstimulated lymphocyte cell cultures of the respective wells. Error bar is standard error of mean.
Western Immunoblot Assay - The immune response was determined by western immunoblot assay (WIA) using the serum which was collected on day 0, 7, 11, 15, and 21 postvaccination. The MAb specific for N protein was also used in the WIA for comparison purposes. Seven days postimmunization two animals from group B showed a marked response (band) to the N protein; by 11-days postvaccination four animals from group B exhibited a marked (response) band to the N protein and three animals from group C showed a very faint band at the same position (Fig. 2). One of the animals from group B also showed a band at the 36 Kd position 11-days postvaccination but a reaction was not apparent at 15 days postvaccination. The remaining animals including controls did not show any visible reactivity to the N protein or other constituents of the virus.

The nature of the immune response was also determined by WIA after challenge. Serum from all animals of group B reacted with the N protein and a marked band was visible 8-days postimmunization. A marked band was also visible at the 36 Kd position, which probably represents the P protein of BRSV strain 375. Three animals from group C also showed a marked response to the N protein and one showed a comparatively faint band at the N protein position; the remaining one animal did not show any band at N protein level, but it showed a marked band at 36 Kd. Serum from control animals was not reactive at 8 days postchallenge (Fig. 3). However, by day 11 most of the control animals exhibited a visible band at the N protein position and one showed a weak response at 36 Kd level (Fig. 4). By day 14, serum from
Figure 2. Analysis of the lambs serum, 11-days postvaccination with washed SMAA complexes by western immunoblot assay. Lane 1-5 represent serum of the group B, immunized with SMAA complexes containing N protein specific MAb (IgM). Lane 6 represent N protein MAb raised against HRSV diluted 1:100 in PBS. Lane 7-11 represent serum of the group C, immunized with SMAA complexes containing N protein specific MAb (IgG2a). Lane 12-15 represent serum of control group A. Lane 15 represents serum of two control animals. The N protein is indicated on the basis of reactivity with The MAb specific for this protein.
Figure 3. Analysis of the lambs serum, 8-days postchallenge with washed SMAA complexes by western immunoblot assay. Lane 1-5 represent serum of the group B, immunized with SMAA complexes containing N protein specific MAb (IgM). Lane 6-10 represent serum of the group C, immunized with SMAA complexes containing N protein specific MAb (IgG\textsubscript{2a}). Lane 11 represent N protein MAb raised against HRSV diluted 1:100 in PBS. Lane 12-16 represent serum of control group A. The N protein is indicated on the basis of reactivity with The MAb specific for this protein. However P protein is indicated on the basis of molecular weight, which is reported to be 36 Kd (Baker et al., 1992).

Figure 4. Analysis of the lambs serum, 11-days postchallenge with washed SMAA complexes by western immunoblot assay. Lane 1-5 represent serum of the group B, immunized with SMAA complexes containing N protein specific MAb (IgM). Lane 6 represent N protein MAb raised against HRSV diluted 1:100. Lane 7-11 represent serum of the group C, immunized with SMAA complexes containing N protein specific MAb (IgG\textsubscript{2a}). Lane 12-16 represent serum of control group A. The N protein is indicated on the basis of reactivity with The MAb specific for this protein. However P protein is indicated on the basis of molecular weight, which is reported to be 36 Kd.
control animals elicited reactivity with N protein but the response was much weaker than serum from the vaccinated groups. Sera from primed animals was reactive with several viral proteins by 14 days post booster immunization and that reactivity became more pronounced by day-21 postchallenge as indicated in Figure 5.

Serum neutralization assay - Neutralizing antibodies could not be detected in sera of all animals prior to vaccination and challenge. By the fifth day following challenge all animals in the primed groups (B and C) had developed neutralizing antibody titers. A dramatic increase in neutralizing antibody titers occurred by day 8 and these persisted through the 21-day experimental period. Most animals in the control group responded (4 of 5) but the response was very low (Tables 2 and 3).

Virus neutralizing antibody titers were higher in serum of animals in group B as compared to group C (Fig. 6). However, the difference was not statistically significant.

<table>
<thead>
<tr>
<th>Group</th>
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<th>SN titer (log₂) distribution of lambs</th>
</tr>
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<tr>
<td>B</td>
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</tr>
<tr>
<td>C</td>
<td>5</td>
<td>- - - - 2 2 1 -</td>
</tr>
</tbody>
</table>
Figure 5. Analysis of the lambs serum, 21-days postchallenge with washed SMAA complexes by western immuno blot assay. Lane 1-5 represent serum of the group B, immunized with SMAA complexes containing N protein specific MAb (IgM). Lane 6-10 represent serum of the control group. Lane 11-15 represent serum of group C, immunized with SMAA complexes containing N protein specific MAb (IgG). Lane 16 represent hyperimmune serum raised against BRSV in sheep. The N protein is indicated on the basis of reactivity with the MAb specific for this protein. However other proteins including $F_0$, $F_1$, $F_2$, P, M and 23 Kd are indicated on the basis of their apparent molecular weights as reported in the literature (Mallipeddi et al., 1990; Baker et al., 1992). There are several other protein bands which are most probably cell band or some viral proteins which are not reported.
Table 3. Group wise SN titer (log 2) (mean±SE) of different groups

<table>
<thead>
<tr>
<th>Group*</th>
<th>Day-0</th>
<th>Day-5</th>
<th>Day-8</th>
<th>Day-11</th>
<th>Day-14</th>
<th>Day-21</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td>0.40±0.25</td>
<td>0.60±0.40</td>
<td>1.40±0.51</td>
<td>1.60±0.51</td>
<td>2.20±0.37</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>3.20±0.49</td>
<td>5.80±0.37</td>
<td>6.20±0.49</td>
<td>6.40±0.68</td>
<td>6.40±0.68</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>2.80±0.37</td>
<td>5.00±0.32</td>
<td>5.20±0.37</td>
<td>5.80±0.37</td>
<td>5.80±0.37</td>
</tr>
<tr>
<td>LSD</td>
<td></td>
<td>1.18</td>
<td>1.13</td>
<td>1.42</td>
<td>1.65</td>
<td>1.53</td>
</tr>
</tbody>
</table>

*Each group had five lambs.

LSD = least significant difference (P=0.05).

Means with same superscript not significantly different based on the LSD.

Serum neutralizing antibody responses of vaccinated and control groups following challenge with heat inactivated BRSV. The serum-virus neutralization titer was the highest dilution of serum in which 50% of the cells in a well were protected from the viral CPE. The results were analyzed by analysis of variance with a 95% confidence level.
Figure 6. Serum neutralizing antibody responses of vaccinated and control groups following challenge with heat inactivated BRSV. The serum-virus neutralization titer was the highest dilution of serum in which 50% of the cells in a well were protected from the viral CPE. The results were analyzed by analysis of variance with a 95% confidence level.
Discussion

In the present study two monoclonal antibodies specific for N protein of BRSV, 65-12 (IgM) and 38-6 (IgG2a), were used to make antibody-antigen complexes by incubating these with BRSV-infected cell lysate. SMAA complexes were prepared by incubating the antibody-antigen complexes with antimouse IgM-agarose or Protein A-agarose beads according to the isotype of the MAbs. The SMAA complexes so formed were washed five times with PBS, suspended in an equal quantity of aluminum hydroxide gel, and used for immunization of two groups of lambs. The immune response was monitored by the lymphocyte transforming assay (LTA) and western immunoblot assay (WIA) and serum neutralization (SN) assay.

Enhanced lymphocyte blastogenic responses occurred on stimulation of cultured lymphocytes from vaccinated animals with viral antigen. Peak responses were noted on day 11 postvaccination in both groups, but of the responses of group B, vaccinated with SMAA complexes containing MAb (IgM), was significantly higher than the group C, vaccinated with SMAA complexes containing MAb (IgG2a). The mean stimulation index of BRSV antigen stimulated cultures significantly increased from 1.47 ± 0.32 before challenge to 3.46 ± 1.64 five days after challenge. Lymphocytes from the lambs were responsive in LTA until 21 days and 11 days postvaccination in group B and C respectively, which indicated that the SMAA complexes containing MAb (IgM) induced higher and prolonged responses than the
SMAA complexes containing MAb (IgG2a). Because of the difficulty in reproducing experimental BRSV infections in calves, studies on the effect of challenge on cellular immune responses are lacking. Recently Ellis et al. reported a significant increase in proliferative responses of cattle lymphocytes to live BRSV by day 7 after primary immunization with a vaccine containing inactivated BRSV. Sharma and Woldehiwet have reported contrasting results regarding BRSV antigen stimulation in vitro after primary infection and reinfection. They did not monitor any significant lymphocyte transformation responses in experimentally-infected lambs up to 21 days after inoculation. However, these lambs when challenged seven months after primary infection showed significantly higher responses to BRSV antigen in vitro than the control lambs.

In this study we also analyzed the humoral response of all animals by WIA. Sera from most of the vaccinates i.e., 80% from group B reacted strongly with the N protein as indicated by a band at the N Protein location and 60% from group C exhibited a very faint band at the same position, 11-days postvaccination. Thereafter, the reactivity determinable by WIA appeared to decline. Similar findings have been reported with N protein expressed in vaccinia by Connors et al. These findings are also to some extent supported by an earlier report which indicated that radioimmunoprecipitating antibodies were induced in BALB/c mice after 21 days of vaccination with N protein expressed in vaccinia virus. None of the lambs developed a SN antibody titer after vaccination.
The findings of this experimentation indicate that SMAA complexes formed with the N protein of BRSV are highly immunogenic in lambs. Further, they are capable of inducing both humoral and cell-mediated immunity. Immunization of mice with solid matrix-antibody-antigen complexes, containing either internal or external structural proteins from the same virus (Simian virus 5) or from different viruses (herpes simplex virus, influenza virus, measles virus and simian virus), have been reported to induce vigorous humoral and cell mediated immune responses.25,26,27,28

A challenge dose of heat-inactivated BRSV 21 days following the priming injection resulted into a far better SN titer in the SMAA complex vaccinated groups than in the control group. Not only did vaccinated animals develop a higher titer but the response developed earlier since all animals seroconverted within five days of challenge. The immune response further increased and peaked by 14 days postchallenge. In comparison, the control group responded very slowly and maximum response was not observed until 21 days postchallenge. The titer was quite low as compared to the vaccinated groups. Among the vaccinated groups, the highest SN titer was observed in the group vaccinated with the SMAA complexes containing MAb (IgM).

These results indicate that vaccination with N protein incorported in SMAA complexes sensitized the lambs and this contributed to a carrier effect resulting into increased SN titer in the vaccinated groups following a challenge with inactivated virus. It appears that primary vaccination induced T and B lymphocytes reactive with
N protein. Consequently when lambs were boosted, because of the presence of induced lymphocytes, there was an increased response to the N protein as compared to unvaccinated lambs. Sensitized helper T lymphocytes also probably provided help to B lymphocytes reactive with other viral proteins. This resulted in an enhanced humoral response to other viral proteins including those involved in virus neutralization.

Antibody-antigen complexes are known to enhance the immune response and have been used for inducing both humoral and cell-mediated immune responses. The antibodies bind to their antigens forming immune complexes and thus promote the ingestion of antigen by monocytes through antibody-monocyte FcR interaction and enhance antigen processing and presentation. Such antibody potentiating effect occurs in vivo and probably plays a significant role in the induction of immune response.

Monoclonal antibodies can be attached to particulate solid matrices and the solid matrix-antibody complexes can then be used to purify the required antigen. The complexes so formed can be used as an immunogen. A major advantage of designing SMAA complexes as vaccines is in the preparation of the immunogen and they permit a relatively simple procedure be used for preparation of the antigen. Since these complexes are to be used as an immunogen, there is no need to use strong denaturing conditions to remove antigen from the complexes. Their property of being relatively stable to prolonged storage can be advantageous in developing countries.
where it may be difficult to store some vaccines. Also antigens derived from virtually any source, be it expression vectors or virus-infected tissue culture cells, can simply be incorporated into SMAA complexes. SMAA complexes do not appear to have been used for inducing immunity against RSV. There would appear to be application of this methodology for immunization against this viral infection. The potential to induce both cell-mediated and humoral immunity to a specific viral protein is encouraging. The approach would allow evaluation of the immunogenicity of various structural components of the virus. The best option for future work would be to prepare SMAA complexes as reported or use N protein expressed in recombinant vaccinia virus. This expressed protein could then be used for preparation of immune complexes. Such complexes could then be used to determine immunogenicity in calves having maternal immunity. These comparative analyses will help to determine the effectiveness of N protein as an immunogen in the presence of maternal immunity and may pave the way to a better vaccine for calves having maternal immunity, and provide a means for protecting young animals from infection with this virus.

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GENERAL SUMMARY

Bovine respiratory syncytial virus (BRSV), an important pathogen of cattle, is considered to be a major contributor to the incidence of respiratory tract disease in nursing beef, feed lot and dairy calves. It causes the greatest morbidity in calves during the first six months of life when maternal immunity is still present. Reinfections are common and difficulties have been encountered in providing protection through live or inactivated vaccines against respiratory syncytial viruses. A major limitation of these vaccines is that maternally-derived immunity to BRSV suppresses an active immune response to vaccination in calves. The basis for this suppression is not well understood. It may result from the blocking effect of the specific anti-viral antibodies.

The purpose of the present investigation was to study the morphogenesis of bovine respiratory syncytial virus (BRSV) strain 375, to generate monoclonal antibodies (MAbs) against its N protein, and to use solid matrix-antibody-antigen complexes containing N protein of BRSV and a MAb to investigate the immunogenicity of the N protein and determine its carrier effect on the subsequent vaccination with heat inactivated virus.

The morphology of BRSV strain 375, originally isolated from nasal secretions of a calf (Lehmkuhl et al. 1979), was observed in Vero cells at a stage when extensive cytopathic effects were present. Electron microscopic examination of ultrathin
sections of BRSV-infected Vero cells revealed not only spherical and filamentous forms of the virus but also bridges between the spherical particles. The bridges did not appear to be arranged at a particular angle. Because of the bridges the virus particles occurred in large aggregates requiring that this factor be taken into account while purifying the virus. Both forms were observed budding from the cell membrane of infected cells. Most of the observed mature particles were extracellular. However a few spherical particles with and without internal structure were also present in intracytoplasmic vesicles. This confirms an earlier report on assembly of virus particles intracellularly in Vero cells infected by a member of the family Paramyxoviridae. Further studies are indicated, using different cell lines to minimize the host cell related variations to arrive at some final conclusion regarding the intracytoplasmic maturation of different strains of BRSV.

A panel of monoclonal antibodies (MAbs), derived from the fusing of the primed BALB/C mice spleenocytes to SP2/O cells, were generated against the N protein of BRSV. Different methods were used to immunize the mice for production of hybridomas and eventually MAbs. The MAbs were characterized by ELISA, a radioimmunoprecipitation assay, indirect immunofluorescence, and epitope mapping. According to the class and subclass of Ig these MAbs can be grouped into four groups. Eight MAbs, two from each group, were used for epitope mapping. The competitive binding assay indicated competing and non competing MAbs. The reaction pattern of the MAbs indicated 4 to 6 antigenic sites on the N protein.
Immune complexes consisting of radiolabeled BRSV and newly generated N protein MAbs, or radiolabeled BRSV and human respiratory syncytial virus N protein MAb were mixed and then electrophoresed together to monitor the electrophoretic mobility of the precipitated N proteins. Both the N proteins moved together showing that they had the same molecular weight (43.4 Kd) and signifying that the generated MAbs were directed against the N protein of BRSV.

The genome of the respiratory syncytial viruses codes ten proteins including N, a structural protein. A number of workers have analyzed the antibody response to N protein of human respiratory syncytial virus (HRSV) in experimental animals with variable results. The general consensus is that the protection induced by N protein of HRSV does not last long and is not as solid as provided by G and F proteins. Keeping in view such reports and our own preliminary studies we considered it possible to use the N protein of BRSV as vaccine during the early period of the calves when they have maternal antibodies against BRSV. But the first thing was how immunogenic the N protein is? Since earlier reports indicated that the N protein is not highly immunogenic we decided to incorporate it in SMAA complexes and use these in lambs to determine it's immunogenicity.

Two monoclonal antibodies 65-12 (IgM) and 38-6 (IgG2a) were used to make solid matrix-antibody-antigen complexes (SMAA). The SMAA complexes so formed were washed five times with PBS, suspended in an equal quantity of aluminum hydroxide gel and used for immunization of two groups of lambs. The immune
response was monitored by lymphocyte transforming assay (LTA), western immunoblot assay (WIA) and serum neutralization (SN) assay. In immunized groups the maximum immune response in terms of lymphocyte blastogenic responses (stimulation index) was observed on day 11 postvaccination. However, the response of animals in group B, vaccinated with SMAA complexes containing MAb (IgM) was significantly higher than the group C, vaccinated with SMAA complexes containing MAb (IgG). The group B animals also exhibited a marked humoral response to N protein in WIA as compared to group C and control animals 11 days postvaccination. None of the animals developed a SN antibody titer after vaccination. Subsequent challenge, 21 days postvaccination, with heat inactivated BRSV resulted in a far better SN titer in the SMAA complexes vaccinated groups than the control group.

The premise for this experimentation was based on a classical immunologic phenomenon observed some 25 years ago and termed the "carrier effect". While the mechanistic basis was not clear at the time of the initial observations of this phenomenon, subsequent experimentation and increased knowledge of the immune system have defined the operational mechanisms. An immune response requires cooperation between two or more populations of cells including for a humoral response an antigen presenting cell, a helper T lymphocyte and a B lymphocyte. The T lymphocyte reacts to immunogenic determinants associated with the carrier moiety of the antigen while the B cell recognizes and responds to haptenic determinants. The latter responds with the production of antibodies but usually only when T cell help is
available. A virus consists of multiplicity of immunogenic and haptenic epitopes and it is possible that a B cell could receive help from T cells responding to a variety of different immunogenic determinants. Consequently, it was theorized that T cells primed to one protein component of a virus might provide enhanced help to B lymphocytes responding to another component of that virus.

The hypothesis for this experimentation was that immunization with a protein of BRSV would prime these animals for subsequent secondary responses to not only the N protein but other structural components of the virus. The results of this experimentation are extremely promising. The N protein was quite immunogenic when administered as an SMAA complex and induced both humoral and cell-mediated responses. These complexes are easily prepared and administered. Secondary or booster immunization with intact, killed virus induced vigorous antibody responses. The nature and degree of this response in previously vaccinated animals as compared to controls is indicative of a secondary immune response. This result was to be expected with the response to N protein but obviously the response extended to other antigenic components of the virus. This was indicated by the level of antibodies in sera as detected by WIA but most importantly, by the dramatic rise in the titers of neutralizing antibodies. Neutralizing epitopes of this virus are associated with the F and G glycoproteins (not N protein) which can be regarded as protective antigens. The important consideration is that animals can be primed for a
response to these antigens without incorporating those antigens in the primary immunogens. Thus, we have confirmed our experimental hypothesis.

The question to be resolved is the potential application of this methodology to immunization of calves with residual maternal antibodies. Available evidence indicates that calves derive antibodies directed against several viral proteins from their dams. However, antibodies directed against the F protein are usually highest in titer with resultant persistence of these antibodies for several months in the blood circulation of the calf. Antibodies to the N protein are present at lower levels and, consequently, do not tend to persist in the calf. This situation should permit immunization of calves at a relatively early age with a vaccine composed of N protein. This could potentially induce some protection against viral infection but also would prime these calves for a secondary immune response. Administration of a second vaccine composed of intact virions could be expected to induce solid protection against infection by the virus.

While this immunization protocol would seem to have great potential, one aspect of the approach could be the basis for a problem. Vaccines containing proteins of foreign species are widely recognized for eliciting hypersensitivity reactions. The SMAA complexes utilized in these studies contained MAbs of mouse origin. The development and use of MAbs of ovine or bovine origin could be a preferred approach.


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