This is to certify that the

thesis entitled

Production and Characterization of Monoclonal Antibodies to Bovine Respiratory Syncytial Virus

presented by

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has been accepted towards fulfillment
of the requirements for

Master degree in *Science*

Department of Large Animal Clinical Sciences

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Date 3/22/93

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO BOVINE RESPIRATORY SYNCYTIAL VIRUS

By

Wendy Jane Underwood

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Large Animal Clinical Sciences

1993

ABSTRACT

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO BOVINE RESPIRATORY SYNCYTIAL VIRUS

By

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Monoclonal antibodies (Mabs) to the SmithKline Beecham Animal Health BRSV^R vaccine strain (375) were produced and characterized by radioimmunoprecipitation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, by virus neutralization, by inhibition of viral-induced fusion, and by isotype. Nineteen hybridomas produced antibodies that were reactive with the F_0, F_1, F_2, N, or P viral proteins of bovine respiratory syncytial virus. Hybridoma 1E72C4 produced Mabs that immunoprecipitated the F_0 glycoprotein, neutralized virus (1:8) in the presence of complement, but did not inhibit fusion. Hybridoma 8B21E7 produced Mabs that immunoprecipitated the F_0, F_1, and F_2 glycoproteins, neutralized virus (1:4) with and without complement, and inhibited fusion. Antibodies from eleven hybridomas immunoprecipitated the N and F_1 viral proteins, one hybridoma immunoprecipitated the N and P protein, and five hybridomas immunoprecipitated the N protein. All Mabs were of the IgG_{2b} subtype with either kappa or lambda light chains.

It gives me great pleasure to dedicate this work

to George and Suzanne Underwood,

my father and my mother,

for their unfailing love, support, and encouragement.

ACKNOWLEDGEMENTS

I would like to extend my sincere and heartfelt gratitude to Dr. John C. Baker, my major professor, for his assistance, guidance, and encouragement during this project. I would also like to extend a special thank you to Dr. Leland F. Velicer for generously making his laboratory and his scientific expertise available for this project. Dr. John M. Kruger and Dr. Robert E. Holland, other members of my committee, deserve thanks for their guidance, suggestions, and input. Our co-investigator, Dr. Maurice A. Mufson, also deserves recognition for his assistance in development of this project as well as characterization of monoclonal antibodies.

Quentin M. McCallum deserves special recognition. Without his technical assistance, perseverance, and unfailing friendship, this project may not have been completed. In addition, I would like to thank Ruth A. Stringer for her technical assistance, encouragement, and support during this study.

Funding for this project was provided by SmithKline Beecham Animal Health.

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LIST OF ABBREVIATIONS

BRSV	-	Bovine respiratory syncytial virus
BM	-	Lectin coated agarose beads incubated with media from BRSV-infected BT cells
BL	-	Lectin coated agarose beads incubated with BRSV-infected cell lysates
$BRSV^R$	-	SBAH $BRSV^R$ vaccine used for immunization of mice
BT	-	Bovine turbinate cells
CF	-	Complement fixation
CPE	-	Cytopathic effect
cpm	-	counts per minute
DISC	-	35-45% sucrose interface of the discontinuous gradient
DMEM	-	Dulbecco's minimum essential medium
EIA	-	Enzyme-linked immunoassay
EMEM	-	Eagle's minimum essential medium
FCS	-	Fetal calf serum
gp(s)	-	glycoprotein(s)
hr(s)	-	Hour(s)

HRSV	-	Human respiratory syncytial virus
HS	-	Horse serum
IFA	-	Indirect immunofluorescent antibody assay
IP	-	Intraperitoneal
kDa	-	Kilodalton
Mab(s)	-	Monoclonal antibody(ies)
PBS	-	Phosphate buffered saline
PEG	-	Polyethylene glycol
PFU	-	Plaque forming units
PI	-	Postinfection
RIPA	-	Radioimmunoprecipitation assay
Rnase A	-	Ribonuclease A
RSV	-	Respiratory syncytial virus
SBAH	-	SmithKline Beecham Animal Health
SDS-PAGE	-	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
VL	-	Virus purified to the linear gradient
VRV	-	Vaccinia recombinant virus
WCC	-	Glutaraldehyde-fixed whole cell culture of BRSV-infected BT cells used to immunize mice

INTRODUCTION

Respiratory syncytial virus (RSV) is a *Pneumovirus* within the Paramyxoviridae family (Table 1);(Stott 85). Human respiratory syncytial virus (HRSV) is the primary cause of pneumonia and bronchiolitis in infants age two to six months (Belshe 83, Chanock 82), while bovine respiratory syncytial virus (BRSV) is an important cause of respiratory tract disease in nursing beef cattle, feedlot calves, and dairy calves (Baker 85,86a,b,c,d, Bohlender 82, Castleman 85, Frey 83, Gillette 85, Harrison 85, Lynch 85, Martin 86, Stott 85). The fact that an effective killed or live-attenuated vaccine for HRSV has not been developed (Belshe 84, Kim 69) has instigated studies concerning the molecular nature of RSV to define important protective RSV antigens and to determine if antigenic variation among HRSV isolates exists. Such information is essential to the development of effective vaccines.

Human respiratory syncytial virus is a negative sense, single stranded, linear RNA virus. The genome of HRSV produces ten mRNA's encoding ten proteins (Table 2);(McIntosh 91, Stott 85). Two major transmembrane glycoproteins (gps), F and G, appear to be important for protective immunity. The F gp is synthesized as a 68 to 70 kDa precursor molecule (F_0) that is proteolytically cleaved into two active disulfide

bonded 48 kDa (F_1) and 23 kDa (F_2) subunits. The F gp promotes viral entry into cells and cell-to-cell spread by fusion of infected cells to uninfected cells (Walsh 83a,85,86). The G gp is associated with viral attachment and is synthesized as a 33 kDa precursor that is extensively glycosylated to yield a mature 90 kDa gp (Levine 87). Five other virion structural proteins have been identified: three nucleocapsid-associated proteins (N, P, and L) and two nonglycosylated internal membrane associated proteins (M and 22 kDa);(Collins 86, McIntosh 90). The remaining proteins 1A, 1B, and 1C (also designated SH, NS1, and NS2, respectively) appear to be nonstructural proteins.

Until recently, HRSV was considered to be a monotypic virus (Beem 60, Bennet 62). Studies conducted during the 1960's, however, suggested antigenic differences among HRSV isolates using postinfection (PI) ferret, rabbit, and guinea pig sera in cross neutralization tests (Coats 63,66a, Doggett 65, Suto 65, Wulff 64). Such differences were not observed when human PI sera was used. Since investigators were unable to demonstrate significant clinical differences in disease despite the presence of antigenic variation among HRSV isolates, it was concluded at this time that clinically relevant antigenic variation did not exist (Coates 63,66a, Hierholzer 79, Prince 85a, Wulff 64).

With the advent of modern molecular techniques, antigenic variation among HRSV isolates has been well documented using polypeptide maps and migration patterns (Cash 77, Gimenez 86, Storch 87), reaction panels of monoclonal antibodies (Akerlind 86, Anderson 85, Gimenez 84,86, Morgan 87, Mufson 85, Norrby 86, Orvell 87, Storch 87, Stott 84b, Tsutsumi 88, Ward 84), and nucleotide sequence divergence analysis (Garcia-Barreno 90, Johnson 87a, Samal 91a,b). HRSV has been divided into two

distinct antigenic subgroups designated A and B (Mufson 85). Antigenic differences were seen in five different structural proteins including F, G (Storch 87), N (Ward 84), M, and P (Gimenez 84, 86) with the most pronounced differences occurring in G gp (Johnson 87a, Mufson 85, Walsh 86b). Additionally, the F_1 and P polypeptides were found to have a consistently higher apparent molecular weight in subgroup A than in subgroup B (Gimenez 86, Morgan 87, Norrby 86). Subsequently, HRSV subgroup A was separated into six subdivisions (Anderson 91), and subgroup B was separated into either two (Akerlind 88) or three subdivisions (Anderson 91, Siqueira 91), while some HRSV isolates could not be classified as either subgroup A or B (Anderson 91).

These findings with HRSV raise several questions with regards to BRSV: namely, is BRSV antigenically distinct from HRSV, and do antigenic subgroups exist among BRSV isolates? Preliminary studies using neutralization tests (Smith 75), peptide maps (Lerch 89,Ward 84), and Mab reaction panels (Baker 92, Orvell 87, Stott 84b, Ward 84) indicate that BRSV is antigenically distinct from HRSV. The BRSV F, N, M, and P proteins showed antigenic cross-reactivity with HRSV, whereas the BRSV G gp showed major antigenic differences using immunoprecipitation and western blot analysis (Lerch 89). Similarly, the deduced amino acid sequence analysis of the F gp of one BRSV isolate (391-2) showed nearly 80% homology to HRSV, while the deduced amino acid homology of the G gp was only 30% with either subgroup A or B of HRSV (Lerch 90,91). While these data indicate that BRSV is a virus that is distinct from HRSV, and that BRSV belongs in a different antigenic group than HRSV, a question remains as to whether antigenic variation exists among BRSV isolates. To date no reports of antigenic

variation within BRSV have been published.

The hypothesis of this project is that antigenic variation does occur among BRSV isolates and that like HRSV the greatest antigenic differences occur in the G gp. To investigate the possibility of antigenic variation among BRSV isolates, a panel of monoclonal antibodies (Mabs) to several selected isolates of BRSV must be produced and reacted with other isolates of BRSV by enzyme-linked immunoassay, indirect immunofluorescence, virus neutralization, inhibition of virus-induced fusion, and radioimmunoprecipitation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. First, a panel of Mabs is produced to one isolate of BRSV and reacted against other BRSV isolates. Based on results, the next isolate of BRSV to produce a panel of Mabs against is selected. Antigenic variation among BRSV isolates can then be defined by reacting many BRSV isolates against these panels of Mabs. The purpose of this study was to produce and characterize a panel of Mabs to a single isolate of BRSV, the SmithKline Beecham Animal Health BRSV^{R} vaccine strain.

Table 1. Genera and species of the Paramyxoviridae family[a].

Genus	Species	Host
Paramyxovirus	Sendai virus	Murine
	Human parainfluenza types 1 - 4	Human
	Canine parainfluenza type 2	Canine
	Bovine parainfluenza type 3	Bovine
	Newcastle disease virus	Chicken
	Mumps	Human
Morbillivirus	Measles virus	Human
	Canine distemper virus	Canine
	Peste de petit ruminants	Ovine/Caprine
	Rinderpest virus	Bovine
Pneumovirus	Human respiratory syncytial virus	Human
	Bovine respiratory syncytial virus	Bovine
	Ovine respiratory syncytial virus	Ovine
	Caprine respiratory syncytial virus	Caprine
	Turkey rhinotracheitis virus	Turkeys
	Pneumonia virus of mice	Murine

[a] Kingsbury DW. Paramyxoviridae and their replication. *In* Fields BN, Knipe DM, Eds, Fundamental Virology (Second Ed). New York:Raven Press, 1991;507-524.

Table 2. Comparison of viral proteins in Human and Bovine Respiratory Syncytial Viruses.

HRSV[a] Molecular Weight (kDa)	Designation	Function	BRSV[b] Molecular Weight (kDa)
250	L	Polymerase	200
80 - 90	G	Attachment	85 - 100
68 - 70	F_0	Fusion	68
48	F_1	Large subunit of F_0	48
43.5	N	Nucleocapsid	42
36	P	Phosphoprotein	34
28.7	M	Matrix	29
22	22 kDa (M2)	Unknown	23
23	F_2	Small subunit of F_0	21
15.6	1C (NS_2)	Nonstructural	15
14.7	1B (NS_1)	Nonstructural	13.5
7.5	1A (SH)	Small transmembrane	11

[a] McIntosh K, Chanock RM. Respiratory syncytial virus. *IN* Fields BN, Knipe DN Eds, Virology (Second Ed). New York:Raven Press, 1990;1045-1072.

[b] Mallipeddi SK, Samal SK, Mohanty SB. Analysis of polypeptides synthesized in bovine respiratory syncytial virus-infected cells. Arch Virol 1990;115:23-36.

LITERATURE REVIEW

Overview

Respiratory syncytial virus (RSV) has been recognized as an important cause of respiratory tract disease in both infants and calves (McIntosh 90, Stott 85). Respiratory syncytial virus was first isolated in 1956 from a laboratory chimpanzee with upper respiratory tract disease and was originally named the "Chimpanzee Coryza Agent" (Morris 56). The following year, an identical virus was isolated from two children in Baltimore with severe pneumonia (Chanock 57a,b). This virus was named human respiratory syncytial virus (HRSV) to connote the formation of multinucleated "syncytial cells" in tissue culture. Human respiratory syncytial virus has since been recognized as the primary cause of pneumonia and bronchiolitis in infants age two to six months (Belshe 83, Chanock 89, Downham 75, Gardner 67, Glezen 73, Kim 73, McIntosh 90).

In 1970, a virus morphologically similar to HRSV was isolated from cattle in Switzerland with respiratory disease (Paccaud 70). This virus was subsequently named bovine respiratory syncytial virus (BRSV) because typical syncytial cytopathology was evident. Bovine respiratory syncytial virus is now recognized as an important cause of respiratory tract disease in nursing beef, feedlot, and dairy calves worldwide (Baker

85,86a,b,c, Bohlender 82, Castleman 85, Frey 83, Gillette 85, Harrison 85, Lynch 85, Martin 86, Stott 85).

Infections due to RSV have also been reported in other species. In 1979, a RSV was isolated from goats (Lehmkuhl 80, Smith 79), and in 1983, the presence of a RSV was reported in sheep (LeaMaster 83). Serum antibodies to RSV have also been reported in dogs (Lundgren 69), cats (Pringle 78), horses (Berthiaume 73), pigs (Doggett 68), deer (Baker 85), and antelope (Baker 85). Unfortunately, little is known about the natural incidence, biology, and epidemiology of RSV in these species.

The fact that an effective killed or live-attenuated vaccine for HRSV has not been developed (Belshe 82, Kim 69) has prompted studies concerning the nature of HRSV to determine if antigenic variation exists among HRSV isolates. In several instances similar studies have been done for bovine, ovine, and caprine RSV isolates. This comparative review of the structural, molecular, clinical, and antigenic properties of HRSV and BRSV (with reference to caprine and ovine RSV when appropriate) addresses questions concerning the relationship of bovine, ovine and caprine RSV to HRSV, the occurrence of antigenic variation among HRSV isolates, and the possibility of antigenic variation among BRSV isolates.

Comparative Features

Classification

Both HRSV and BRSV belong in the Paramyxoviridae family. Viruses of this family primarily infect warm blooded mammals and cause important diseases in man and animals including measles and mumps in humans, canine distemper in dogs, and

rinderpest in cattle (Table 1). Human and bovine RSV belong to the genus *Pneumovirus*, which together with the genus *Paramyxovirus* and the genus *Morbillivirus* make up the Paramyxoviridae family. The division into three genera was based on differences in possession of two viral particle activities, hemagglutination and neuraminidase (Kingsbury 91). Members of the *Paramyxovirus* genus agglutinate mammalian erythrocytes and have neuraminidase activity, while the *Morbillivirus* members hemagglutinate but do not have neuraminidase activity (Kingsbury 91). Pneumoviruses differ from others in the family in that they lack hemagglutination and neuraminidase activity, possess a narrower nucleocapsid, and have a unique number and order of genes and gene products (Collins 86, Kingsbury 91). The pneumovirus "type" species is HRSV. In addition to HRSV and BRSV, goat and sheep RSV, pneumonia virus of mice (Melnick 71), and turkey rhinotracheitis virus (Yu 92) belong in this group.

Paramyxoviruses have enveloped virions with helical nucleocapsids that contain a linear, single stranded, nonsegmented, negative-sense RNA genome (McIntosh 90, Stott 85). Morphologically, virions appear as round or pleomorphic forms, 150 to 300 nm in diameter, with an outer membrane studded by projections 12 to 15 nm in length and 10 nm apart (which represent the F and G viral proteins);(Belsche 84, McIntosh 90, Stott 85). Replication occurs within the cytoplasm and the virion matures by budding from the cytoplasmic membrane.

Molecular Biology

The HRSV genome contains approximately 15,000 nucleotides with one promotor, is transcribed as a single unit, and encodes ten mRNA's and ten proteins

(Table 2);(Collins 86, McIntosh 90). The viral envelope contains two major glycoproteins (gps), F (fusion) and G (attachment). The F gp is a typical paramyxovirus fusion protein that promotes viral entry into cells and cell-to-cell spread by fusion of infected cells to uninfected cells (Trudel 87, Walsh 83a,85,86). The F gp is synthesized as an inactive 68 to 70 kDa precursor polypeptide (F_0) that must be proteolytically cleaved into two disulfide-bonded 48 kDa (F_1) and 23 kDa (F_2) subunits to mediate fusion (Walsh 85). The G gp has been associated with viral attachment and is synthesized as a 33 kDa precursor polypeptide that is extensively glycosylated to yield a mature gp of 80 to 90 kDa (Levine 87, McIntosch 90, Walsh 84). A soluble form of G is released into tissue culture fluids of intact RSV-infected cells (Hendricks 87,88).

In addition to the F and G gps, HRSV has five other structural proteins. The internal side of the membrane is lined by a nonglycosylated 28.7 kDa matrix (M) protein. A 22 kDa protein (previously designated M2) is also associated with the internal side of the envelope, but the function of this protein is unknown. The internal helical nucleocapsid is a complex of three proteins: N (nucleoprotein), P (phosphoprotein), and L (large polymerase);(Collins 86, Huang 85, McIntosh 90). The N protein serves a structural and protective function and has a molecular weight of 43.5 kDa. The P protein has a molecular weight of 32 kDa and is heavily phosphorylated, while the L protein has a MW of 250 kDa. The P and L proteins are believed to play a role in transcription or replication of viral RNA (McIntosh 90).

Another protein, 1A (also designated SH), has recently been shown to be a 7.5 kDa transmembrane protein present in both glycosylated and nonglycosylated forms

(Huang 85, McIntosh 90). The remaining two proteins, a 14.7 kDa protein (also designated 1B or NS1) and a 15.6 kDa protein (also designated 1C or NS2), appear to be nonstructural proteins since both are found in infected cells but not in the virion itself (Collins 86, Huang 85, McIntosch 90).

Recently two strains of BRSV have been examined at the molecular level (Lerch 89,90,91, Mallipeddi 90). In one study (BRSV isolate 391-2), nine proteins specific to BRSV-infected cells were identified that corresponded to nine HRSV proteins (Lerch 89). In addition, a tenth mRNA was identified by Northern blot analysis indicating that like HRSV, BRSV has ten mRNA's encoding ten proteins (Lerch 89). In another study (BRSV isolate A51908), ten BRSV viral specific proteins were identified by radioimmunoprecipitation (RIPA) with hyperimmune rabbit antiserum followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of BRSV infected cell lysates: a 200 kDa (L), a 85-100 kDa (G), a 68 kDa (F), a 42 kDa (N), a 34 kDa (P), a 29 kDa (M), a 23 kDa (M_2), a 15 kDa (probably 1C), a 13.5 kDa (probably 1B), and an 11 kDa protein (probably 1A);(Mallipeddi 90). A 48 kDa and a 21 kDa gp were also detected which probably represent the F_0 cleavage products (Mallipeddi 90). The BRSV F, N, M, and P proteins showed antigenic cross-reactivity with their HRSV counterparts, while the BRSV G gp showed no antigenic cross-reactivity with the HRSV G gp by RIPA SDS-PAGE, Western (immuno-) blot analysis, and enzyme-linked immunoassay (EIA);(Baker 92, Lerch 89,90, Orvell 87).

Clones of cDNA have been prepared and used to identify the 10 HRSV-specific mRNA's that code for the 10 polypeptides, and the complete nucleotide sequence has

(Huang 85, McIntosh 90). The remaining two proteins, a 14.7 kDa protein (also designated 1B or NS1) and a 15.6 kDa protein (also designated 1C or NS2), appear to be nonstructural proteins since both are found in infected cells but not in the virion itself (Collins 86, Huang 85, McIntosch 90).

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Clones of cDNA have been prepared and used to identify the 10 HRSV-specific mRNA's that code for the 10 polypeptides, and the complete nucleotide sequence has

been determined for the 10 genes (Collins 86, McIntosh 90, Stec 91). For BRSV, the complete nucleotide sequence of the genes encoding the G, F, N, M, 1A(SH), and P viral proteins have been determined (Lerch 90, Mallipeddi 92, Samal 91a,b, Walravens 90, Zamora 92).

Epidemiology

HRSV and BRSV share epidemiological, clinical, and pathological features as well as structural and molecular features.

Infections by HRSV and BRSV occur worldwide and have a seasonal periodicity. Reports indicate that RSV has been isolated from humans and cattle in Europe (Paccaud 70, Peacock 61), Asia (Inaba 72, Suto 65), and America (Chanock 57, Smith 74). In temperate areas, epidemics develop in midwinter for both HRSV and BRSV, but infection can also occur in the summer (Baker 85, Stott 85).

The incidence of infection with HRSV and BRSV is high. Approximately half of all infants become infected with HRSV in the first year of life, while the other half become infected during the second year of life (Kim 73, Stott 85). Although the exact incidence is unknown, infection with BRSV is apparently common. Surveys of BRSV antibody prevalence in cattle in the United States indicate that 60 to 80% of cattle have serum antibody titers (Baker 85).

Although infections with HRSV and BRSV occur primarily in the young, reinfections can occur at any age. HRSV infection rates peak in infants at two months of age and gradually decline by two years of age (Glezen 81, Parrott 73). With BRSV,

the most severe disease occurs in calves from one to three months of age (Harrison 85, Kimman 88). Primary infections can also occur in adult humans and cattle (Hall 78, Baker 86a,b). Repeated infection commonly occurs in humans and cattle, but the severity of disease generally declines as infection shifts from the lower respiratory tract to the upper respiratory tract (Baker 85,86a,b, Cooney 75, Glezen 78, Hall 76,78, Henderson 79, Lehmkuhl 79, Rosenquist 74, Stott 85).

Maternally derived passive immunity does not prevent infection in either infants or calves, but it does modulate the severity of disease (Baker 86a,b,c, Belknap 92, Glezen 81, Kimman 88, Rosenquist 74). The infant has maternally derived antibody to HRSV during the first six months of life, and infants within this age range appear to shed less virus in the presence of maternal antibodies (Kapikian 69, Stott 85). In addition, a relative sparing of severe disease in infants less than three weeks old has been reported (Parrott 73). There is also a relative sparing of disease due to BRSV in calves less than two weeks of age (Kimman 88). The same study indicated that the incidence and severity of BRSV infections was inversely related to the level of BRSV-specific maternal antibody. More recently, a protective role for passively derived antibody was experimentally demonstrated in calves (Belknap 92). Based on clinical parameters, blood gas measurements, and lung lesions, this study found that maternal antibodies modify the severity of BRSV disease.

Clinical Signs

Clinical signs of HRSV and BRSV infection are similar and are usually confined to the respiratory tract. Infections in both infants and calves are characterized by the sudden onset of fever, hyperpnea, lethargy, rhinitis, and cough (Baker 85, Belshe 84). In severe disease, crackles, rales, and rhonchi are frequently detected on auscultation (Baker 85, Belshe 84). Clinical diseases produced by RSV in both infants and calves include bronchiolitis, pneumonia, and bronchitis (Baker 85, Belshe 84).

Pathologic Lesions

Pathologic lesions caused by HRSV and BRSV are also similar. Respiratory syncytial virus typically infects epithelial cells of the respiratory tract causing epithelial cell swelling and loss of cilia (Baker 85, Belshe 84). These luminal epithelial cells eventually die and are shed into the bronchi causing obstruction of the airways. In infants, this may cause trapping of air and hyperinflation of the lung (Belshe 84). In cattle, a diffuse interstitial pneumonia occurs that is characterized by subpleural and interstitial emphysema and edema (Baker 85).

Infections by HRSV and BRSV produce similar histologic findings. As mentioned previously, the fusion protein (F gp) of RSV mediates fusion of infected cell membranes with cell membranes of adjacent cells. The fused cells form multinucleated syncytial cells that are evident on histologic examination of bronchiolar epithelium and lung parenchyma (Baker 85, Belshe 84). Eosinophilic intracytoplasmic inclusion bodies are sometimes present within these giant cells and are thought to be composed of dense intertwining nucleocapsids (Baker 85, Belshe 84). In addition, both viruses cause

lymphocytic bronchiolitis and bronchiolar occlusion, parenchymal inflammation, and alveolar exudation (Aherne 70, Baker 85, Snuffin 81, Stott 85, Werdin 85).

Immune Response

Despite numerous studies of humoral and cell-mediated immunity to HRSV in laboratory animals, the immune mechanisms that underlie the balance between protection, disease, and recovery remain poorly defined (Nicholas 90). Nonetheless, this work has allowed initial definition of proteins important in the immune response to RSV. In HRSV-infected children, antibodies are primarily induced against F, N, and possibly G, whereas in BRSV-infected calves, F and N appear to be the most important in terms of humoral immunity (Gimenez 87, Levine 88, Murphy 86, Westenbrink 89). In both humans and cattle, antibodies may also be directed against the L, P, M, 22K and 1A proteins (Levine 88, Ward 83, Westenbrink 89).

Immunization of cotton rats and mice with vaccinia recombinant virus (VRV) vectors indicate that F and G gp are the major antigens that protect against challenge exposure and that these two proteins also produce neutralizing antibodies (Elango 86, King 87, Olmstead 86, Stott 86, Walsh 87a, Wertz 87). Those animals immunized with VRV-F were better protected than those immunized with VRV-G, and immunization with VRV-N showed less protection than that obtained with either VRV-F or VRV-G (King 87, Olmstead 86, Stott 86,87, Walsh 87a, Wertz 87). Investigation of the protective immune response in mice to nine HRSV proteins (all except the L) expressed individually in VRV concluded that F and G gps were most important for protective immunity (Connors 91).

Investigations of cell mediated immunity to HRSV in mice using vaccinia recombinant virus vectors revealed that the 22 kDa protein (M2) was strongly recognized by cytotoxic T-cell lymphocytes and that the F and N proteins were recognized weakly in comparison (Bangham 86, Nicholas 90, Openshaw 90, Pemberton 87). The P and G proteins were recognized little or not at all by cytotoxic T-cell lymphocytes (Bangham 86). The 1A protein has been shown to contain two T-cell stimulating sites (Nicholas 90). Limited research has been conducted on the cell mediated immune response to BRSV infection. Most studies involve the identification of a cell mediated response in calves post-vaccination based on leucocyte migration-inhibition or lymphocyte transformation tests and do not describe the specific role of viral proteins (Field 84, Taylor 87).

Antigenic Variation

Although HRSV and BRSV share many similarities in structural, molecular, and clinical properties, antigenic differences have been found among HRSV isolates, among BRSV isolates, and between RSV of different species.

Cross neutralization. HRSV was originally considered to be a monotypic virus (Beem 60, Bennet 62). Early studies, however, found antigenic differences between two isolates of HRSV (Long and CH 18537) using postinfection ferret, rabbit, and guinea pig sera in cross neutralization tests (Coates 63,66a, Doggett 65, Suto 65, Wulff 64). Following primary inoculation, animals inoculated with the Long isolate developed a four-fold higher neutralizing antibody titer against the homologous isolate than against the heterologous CH 18539 isolate. This difference in antibody titer was considered

significant and could not be explained by differences in antibody avidity (Coates 63). However, when human postinfection sera was used, differences between isolates were not confirmed. Researchers hypothesized that nonhuman mammals may have a more specific antibody response, that HRSV may have a broad antibody response, or that recurrent infant infection may obscure antigenic differences in isolates (Coates 63, Wulf 64).

Doggett, *et al* (68) were the first to observe that HRSV could be neutralized by bovine convalescent serum. Paired serum samples from cattle with respiratory disease showed a rising titer of an "RS virus-inhibitor factor" (Doggett 68), and, as mentioned previously, two years later the related bovine RSV was isolated in Switzerland (Paccaud 70). In 1975, however, BRSV (FS1-1 isolate) was found to be antigenically distinct from HRSV (Long isolate) by cross neutralization tests using rabbit hyperimmune antisera (Smith 75).

Complement fixation (CF). Subsequent attempts to define antigenic variation in HRSV involved CF tests using sera from ferrets infected with different isolates of HRSV. Since soluble CF antigens could be separated from the viral particle by centrifugation and collection of the supernatant, the existence of an antigen separable from the virus particle made it possible to compare isolates by a technique other than neutralization (Coates 63,66b, Forsyth 66,70). When CF antigens prepared from the Long and CH18537 isolates were tested in comparison with serum from homologous and heterologous infected ferrets, no differences between CF antigens prepared from the two isolates were noted (Coates 63, Wulff 64). It was concluded that HRSV isolates have

common CF antigens. Thus, HRSV isolates could be differentiated on the basis of cross neutralization tests but not on the basis of CF tests. No CF studies have been done with BRSV.

Tissue culture host range. Human respiratory syncytial virus and BRSV were further differentiated based on the ability to infect tissue culture cells of different species (Lerch 89, Matumoto 74, Paccaud 70, Pringle 78, Stott 85). Most studies indicate that BRSV has a narrower host range than HRSV (Lerch 89, Stott 85). However, one study found that the NMK7 isolate of BRSV replicated in bovine, hamster, swine, and primate cells, whereas the Long isolate of HRSV replicates only in bovine and primate cells (Matumoto 74). Other researchers have been unable to repeat these findings using other isolates of BRSV (Lerch 89, Paccaud 70, Pringle 78).

Monoclonal antibodies. Early efforts to obtain a definitive description of the HRSV genomic structure and to identify all of the gene products were severely hindered by difficulties with propagation and purification of RSV *in vitro*. The use of panels of Mabs reacting to various isolates of HRSV by immunoblot, immunofluorescence, immunoperoxidase staining, EIA, and RIPA SDS-PAGE analysis led to a more complete understanding of antigenic variation in RSV (Fernie 82, Walsh 83). Two distinct subgroups of HRSV, designated A and B, were identified (Akerlind 86, Anderson 85, Gimenez 84,86, Hendry 86, Morgan 87, Mufson 85, Norrby 86, Storch 87, Tsutsumi 88, Ward 84). The prototype for subgroup A was designated the Long isolate, and the prototype for subgroup B was designated the CH 18537 isolate. Antigenic differences were noted in five different structural components (F, G, M, P and N), with the most

pronounced differences being observed in the G gp (Mufson 85).

Subsequently, both subgroups A and B of HRSV have been further divided into subtypes based on HRSV Mab reaction panels using immunofluorescence, EIA, and RIPA SDS-PAGE (Akerlind 88, Storch 91). Subgroup B of HRSV was divided into two subtypes, B_1 and B_2, with the major difference residing in the G and P proteins (Akerlind 88). Recently, six subdivisions have been identified for subgroup A, and three subdivisions have been identified for subgroup B (Anderson 91). Furthermore, some HRSV isolates were identified that could not be classified as either subgroup A or B (Anderson 91).

Studies have confirmed that BRSV is antigenically distinct from either subgroup of HRSV based on reaction panels of HRSV Mabs against BRSV isolates (Baker 92, Orvell 87, Stott 84, Ward 84). Antigenic differences in at least five major viral proteins (F, N, M, and P) were found between BRSV and HRSV, although BRSV does have shared epitopes with HRSV in each of these proteins (Orvell 87). The greatest antigenic differences were observed in the G gp since none of the HRSV subgroup A or B Mabs to the G gp reacted with any of the BRSV isolates (Baker 92, Orvell 87, Taylor 84). These findings suggest that BRSV belongs in a different group than HRSV (Baker 92). The division of HRSV into two subgroups and the placement of BRSV into a separate group was supported by peptide mapping studies (Ward 84).

Studies comparing BRSV isolates have been limited. An early study indicated that no antigenic differences were observed between three isolates of BRSV when tested with the Mabs to either subgroup of HRSV by immunofluorescence, EIA, and RIPA SDS-

PAGE (Orvell 87). In the same study, no antigenic differences were found between the three bovine isolates and a caprine isolate. Although a more recent study of nine BRSV isolates showed different patterns of reactivity to Mabs against HRSV by EIA and RIPA SDS-PAGE, it was not possible to categorize BRSV isolates into distinct subgroups on the basis of EIA findings alone (Baker 92).

Protein analysis by gel electrophoresis. Attempts to further identify the antigenic characteristics of RSV proteins involved protein analysis by SDS-PAGE. The apparent molecular weight of the P protein and the F_1 and F_2 cleavage products of the F_0 gp were found to be consistently different between the two HRSV subgroups by SDS-PAGE analysis (Cash 77, Gimenez 86, Mufson 85, Norrby 86). In subgroup B, the F_1 and P proteins were lower in apparent molecular weight than subgroup A, and the F_2 protein was higher in apparent molecular weight (Norrby 86). A recent study correctly subgrouped 54 of 56 HRSV isolates into subgroup A and all 19 HRSV isolates into subgroup B based on differential P protein mobility in one-dimensional SDS-PAGE (Walpita 92). These results suggest that P protein mobility is a stable characteristic and that P protein mobility may be used to accurately identify subgroups (Walpita 92).

Studies concerning P protein mobility in BRSV have been minimal. Because the polypeptide profiles of BRSV isolates appeared denticle, an early report considered antigenic variation in BRSV to be limited (Cash 77). However, this study compared only two isolates. A recent study of nine BRSV isolates, however, indicated that three isolates have P proteins with a consistently lower molecular weight (34 kDa) than six other BRSV isolates with a higher molecular weight P protein (36 kDa);(Baker 92).

Most authors agree that sequence variation fails to explain the variation in P mobility (Johnson 90, Walpita 92). The presence of acidic domains causing conformational changes in the P protein has been suggested as causing differences in P protein mobility (Pakas 91). Others speculate that changes in P protein mobility may be due to differences in posttranslational processing of the protein (Johnson 90).

Epitope mapping. Monoclonal antibodies can be used to topographically map epitopes and to identify antigenic diversity by competitive binding assays (Anderson 86). Several antigenic sites were found on both the HRSV F and G gp (Anderson 86, Trudel 86, Walsh 86). Four to five antigenic sites have been identified on the F protein, of which three to four are involved in neutralization (Beeler 89, Kimman 90, Orvell 87, Samson 86, Trudel 86, Walsh 86). At least two of these neutralization sites also mediate fusion inhibition, and Mab-resistant mutant studies indicate that several epitopes exist within these neutralization sites (Beeler 89). Those antibodies that bound to the F gp and neutralized virus were not able to identify strain variation, but those F gp Mabs that did not neutralize virus were able to distinguish between RSV isolates (Long, A2, 8/60) (Anderson 86, Tsutsumi 87, Walsh 86).

Epitopes found on the G gp showed the greatest antigenic diversity among HRSV isolates (Anderson 86, Kimman 90, Storch 87, Tsutsumi 87, Walsh 86). The G gp of HRSV subgroup A contained three antigenic sites, at least two of which neutralize virus. The G gp of HRSV subgroup B contains at least two antigenic sites, both of which neutralize virus (Anderson 86, Kimman 90, Tsutsumi 87, Walsh 89). Although not all Mabs directed to these sites on the G gp neutralized virus, at least one neutralizing site

was shared by both subgroups (Anderson 86, Kimman 90, Tsutsumi 87, Walsh 89). Competitive binding assays have not been performed using BRSV Mabs against BRSV isolates.

Nucleotide sequence analysis. To further delineate the molecular basis of antigenic variation, it became important to determine whether monoclonal and neutralizing antibody specificity was due to extensive nucleotide sequence divergence or to variation within a limited set of antigenic sites (Johnson 87). When the nucleotide sequences of different HRSV isolates were compared, extensive sequence divergence was found in the G gene, which shared only 53% amino acid identity between subgroups, whereas the homology for other proteins was much higher (76% homology for 1A, 87% homology for 1C, 89% homology for F, 96% homology for N);(Johnson 87a,b,88a,b). Within the same subgroup, however, variation was more restricted (94% homology for G, 98% homology for F, and 98% homology for P);(Baybutt 87, Johnson 87a,b,88a,b, Lambden 85, Lopez 89).

Sequence comparisons have been made between BRSV (391-2, RB 94, A51908) and subgroups A and B of HRSV (RSS-2, A2, and CH18537). The F gp of BRSV and HRSV share an 83 to 84% amino acid homology (Lerch 91, Walravens 90). The nucleotide sequence and the deduced amino acid sequence of the major nucleocapsid protein of BRSV (A51908) showed a 93.3% homology with HRSV (A2, CH18537) at the amino acid level (Samal 91a), while the M protein showed a 74% homology (Samal 91b). Comparison of the P protein sequences of BRSV (A51908, FS-1) with HRSV subgroup A (A2) and B (18537) revealed a 74% homology at the nucleotide level and an

81% identity at the amino acid level (Mallipeddi 92). In addition, an 80% identity at t amino acid level exists for the 22 kDa (M2) protein between BRSV and HRSV (Zamc 92). However, the BRSV G gp (391-2 isolate) amino acid sequence showed only 29 30% amino acid identity with the G gp of HRSV (A2 isolate);(Lerch 90). In additic a low overall homology (38%) at both the nucleotide and amino acid levels was observ between BRSV (A51908) and HRSV (A2, CH18537) in the 1A protein (Samal 91b).

Ribonuclease A mismatch cleavage analysis. Ribonuclease A (Rnase mismatch cleavage analysis has been used to examine the extent of genomic diversi among HRSV and BRSV isolates. The method takes advantage of the resistance double-stranded RNA to cleavage by Rnase A. A [^{32}P]-labeled RNA probe (transcribe from HRSV G gp cDNA) is incubated under appropriate conditions with the RNA bei analyzed. Hybridization of the target RNA with the probe protects homologous regio of the probe from ribonuclease cleavage, while areas of mismatch are susceptible cleavage. Epidemiologically related strains (from coinfected twins and from infar infected during a nosocomial outbreak at an extended care facility) had identic fingerprints, but extensive variation was found within HRSV subgroup A. Although t Rnase A mismatch results are consistent with both sequence data and reactivity of vi isolates with Mabs in that two distinct subgroups of HRSV exist (Christina 90, Mye 85, Storch 89), more genetic heterogeneity was observed among HRSV isolates of t same subgroup than was previously expected (Christina 90, Myers 85, Storch 89).

Preliminary Rnase A mismatch studies have been done with bovine, ovine, a caprine RSV (Duncan 91). A radiolabeled anti-sense riboprobe to the BRSV (3

isolate) G gp gene was produced and cross-hybridized with RNA extracted from ce infected with various RSV isolates. Subjection of the RNA-RNA heteroduplexes Rnase A mismatch analysis suggested that heterogeneity does occur among BRS isolates, that caprine isolates are more closely related to BRSV isolates, and that HRS subgroups A and B and ovine RSV are more divergent from BRSV (Duncan 91).

cDNA nucleic acid hybridization assay. More recently, a cDNA nucleic ac hybridization assay has been utilized to determine subgroup designations of individu isolates of HRSV (Sullender 90b). This technique takes advantage of the nucleoti sequence diversity of the G protein gene to develop a nucleic acid hybridization ass capable of establishing subgroup classification. Virus-infected cells are fixed nitrocellulose with glutaraldehyde. Viral RNA's are then hybridized with [^{32}P]labele cDNA clones that correspond to a portion of the extracellular domain of the G gp either HRSV subgroup A or B. Using this method, subgroup assignment of over 4 HRSV isolates were found to be in agreement with the results of subgroup determinatio based on reactivities with Mabs (Sullender 90b). Application of this technique to BRS isolates has not been reported.

The cDNA hybridization assay has some advantages over the use of Mabs a Rnase A mismatch analysis in determining antigenic variation because the cDN hybridization assay is less sensitive but still able to differentiate subgroup delineatio The specificity of Mabs is such that a single amino acid change in a viral gp can resu in loss of reactivity with a Mab (Laver 79). Anti-G Mab escape mutants (HRSV isolat selected by growth in the presence of an anti-G Mab) not only lost reactivity with

group of Mabs against the G gp, but also failed to react with polyclonal sera against whole virus (Garcia-Barreno 89). Sullender, *et al* (90b) suggested that this capacity for antigenic change may explain why investigations which utilize a limited number of Mabs are unable to assign a subgroup designation to all HRSV isolates (Hendry 88, Russi 89). Since the Rnase A mismatch cleavage technique has been shown to be very sensitive for detecting small changes in RNA sequences not detectable by Mabs, including single-base substitutions (Myers 85), it may prove to be too sensitive a technique to distinguish subgroup variation. The cDNA hybridization approach to RSV subgroup determination has the advantage of providing broad-based discrimination of HRSV subgroups based on nucleic acid homology without interference from minor antigenic differences detected by Mab reaction panels and Rnase A mismatch assays (Sullender 90b).

Restriction endonuclease mapping. More recently, a novel method for rapid identification of HRSV subgroups has been developed that utilizes the polymerase chain reaction followed by restriction endonuclease mapping. Viral RNA is extracted from clinical specimens or from tissue cultures; a cDNA copy is made and amplified using the polymerase chain reaction; and, the polymerase chain reaction products are digested with restriction endonucleases and analyzed by SDS-PAGE. Analysis of N and G gene restriction patterns allowed eighty-six isolates of HRSV to be classified by subgroup and, in the case of subgroup A isolates, into six lineages circulating worldwide (Cane 91,92). Although the method is simple, rapid, and relatively inexpensive, the methodology is more discriminating than analysis using panels of monoclonal antibodies (Cane 92). However, like Rnase A mismatch cleavage analysis, restriction endonuclease mapping

may prove to be too discriminating.

Stability of Antigenic Variation

Several lines of evidence indicate that antigenic variation is a stable characteristic. First, reaction patterns to Mabs were not changed by multiple passages in tissue culture (Anderson 85). Second, differences in epitopes as defined by Mabs were found on at least two major proteins indicating that strain variation is not an occasional mutational event (Anderson 85, Mufson 85). Furthermore, reassortment events, as which occurs with certain orthomyxovirus with segmented genomes, is unlikely to occur with a linear nonsegmented genome (Mufson 85, Prince 85). Third, isolates belonging to the same subgroup obtained 20 years apart give similar reaction patterns (Anderson 85). Fourth, examination of intergenic and non-coding sequence regions of viral RNA between HRSV subgroups indicates that extensive divergence has occurred (Johnson 88). Finally, Rnase A mismatch results suggest that subgroups may evolve by sequential accumulation of genetic changes with time but that discrete stable variants may exist (Christina 90, Storch 89).

Clinical Importance of Antigenic Variation

Early Investigations

Early investigators were unable to demonstrate significant clinical differences in morbidity associated with different isolates despite the presence of strain variation (Coates 66a, Hierholzer 79, Prince 85a, Wulff 64). Examination of paired isolates from successive infections of the same patient suggested that antigenic variation was not the

cause of reinfection (Beem 67, Coates 66a). Since reinfection could occur with the same serotype, researchers speculated that little selective pressure existed for new serotypes to emerge and replace old serotypes (Coates 66). Indeed, newly identified isolates (CH 18539) did not appear to replace other HRSV isolates or assume dominance (Coates 66a). Sequential changes in viral antigens identified from serial annual outbreaks did not appear to occur (Monto 75). In addition, since cotton rats immunized with one isolate were protected when challenged with a different isolate, it was concluded that clinically relevant viral antigenic variation did not exist (Prince 85b).

Epidemiology

Recognition of HRSV subgroups, however, raised key epidemiological guestions: 1) does reinfection represent a new infection with a different subgroup, 2) does a difference exist in subgroup disease severity, and 3) do components of both subgroups need to be included in a vaccine (Mufson 85)? Several studies have shown that both subgroups may be present simultaneously in a single epidemic, with subgroup A occurring more frequently than subgroup B (Akerlind 86, Freymuth 91, Hendry 86, Monto 89, Mufson 87, Tsutsumi 88). However, temporal and geographical clustering can occur as well as variability in subgroup dominance (Hendry 86, Mufson 87, Tsutsumi 88). Although second infections with strains of either subgroup does not potentiate illness (Mufson 87), there is some indication that primary infection with subgroup A produces more severe disease than subgroup B (McConnochie 90, Mufson 91, Taylor 89). Most notably, it was found that subgroup A provided some temporal protection from reinfection with homologous HRSV isolates, but did not provide equal

protection from reinfection with heterologous isolates (Mufson 87, Waris 91).

Immunity

Antibody responses to primary infection with HRSV are both subgroup speci and subgroup cross-reactive. The HRSV F gp has been identified as the most import antigen in inducing cross-protective immunity (Olmstead 86, Stott 87). In contrast, t antibody response to G is mostly subgroup specific (Hendry 88, Johnson 87b, Muelena 91, Stott 87, Sullender 90a). A recent molecular analysis of the attachment protein subgroup B HRSV found that 51% of the G-gene nucleotide changes observed amo isolates resulted in amino acid coding changes in the G protein (Sullender 90a). It w suggested that a selective pressure for change is exerted on the G protein and that t pressure was most likely exerted by the host antibody response. These results, a results of antibody response to primary infection and immunization of cotton ra indicate that both F and G gps are important to immunity and that components of the gp from both subgroups need to be included in a vaccine to ameliorate RSV morbid from both HRSV subgroups (Hendry 88, Sullender 90a).

Summary of Antigenic Variation

In summary, evidence gathered so far suggests that antigenic variation does occ among HRSV isolates, that BRSV belongs to an entirely different group than HRSV, a that antigenic variation may occur among BRSV isolates. First, the majority of evider indicates that HRSV can be divided into two subgroups based on cross neutralizati tests, reaction panels using Mabs, protein analysis by gel electrophoresis, epito

mapping, nucleotide sequence analysis, Rnase A mismatch cleavage analysis, cDNA nucleic acid hybridization analysis, and restriction endonuclease mapping. The majority of antigenic variation between HRSV subgroups occurs in the G gp.

Second, several studies indicate that BRSV belongs to a different group than HRSV and that ovine RSV probably belongs in a group separate from either HRSV or BRSV. HRSV and BRSV infect tissue culture cells of different species (Matumoto 74, Stott 85). Using reaction panels of Mabs, HRSV anti-G Mabs failed to react against BRSV G gp indicating that BRSV probably belongs in a group different from either subgroup A or B of HRSV (Baker 92). Nucleotide sequence data of the BRSV G gp revealed only a 29 to 30% homology to the G gp of either subgroup of HRSV (Lerch 90). Rnase A mismatch cleavage analysis supports evidence that BRSV is distinct from HRSV and that human, bovine, and ovine RSV should be placed in a different groups.

Third, consistent variation in P polypeptide migration patterns between BRSV isolates suggests that BRSV can be divided into at least two subgroups (Baker 92). Sequence data support the findings of polypeptide migration maps and Mab reaction panels that antigenic variation probably does occur among BRSV isolates. One method to define the occurrence of antigenic variation among BRSV isolates is to produce Mabs to several isolates of BRSV, and then to react those Mabs with other isolates of BRSV by EIA, indirect immunofluorescence, virus neutralization, inhibition of virus-induced fusion, and RIPA SDS-PAGE. First, a panel of Mabs is produced to one isolate of BRSV and reacted against other BRSV isolates. Based on results, the next isolate of BRSV to produce a panel of Mabs against is selected. Antigenic variation among BRSV

isolates can then be defined by reacting many BRSV isolates against these panels of monoclonal antibodies.

Other methods to define antigenic variation among BRSV isolates include Rnase A mismatch cleavage, cDNA hybridization, and restriction endonuclease analysis. These methods, especially Rnase A mismatch cleavage analysis, are very sensitive and are able to distinguish small changes in nucleotide base arrangement. One reason to emphasize the Mab approach is that antigenic variation is defined on the immunological level, whereas other approaches define antigenic variation on the nucleic acid level.

OBJECTIVES

The long term goal of this project is to produce and characterize Mabs to several isolates of BRSV; to create reaction panels of Mabs against which other BRSV isolates can be tested; and, to use these Mabs to define antigenic variation among BRSV isolates. The specific aims of this study were:

1) to develop methods of preparing BRSV for the immunization of mice,

2) to develop methods to screen hybridomas for the production of antibodies specific to BRSV viral proteins,

3) and, to produce and characterize a panel of Mabs to the SBAH BRSV^{R} vaccine strain of BRSV.

MATERIALS AND METHODS

Virus and Cells

The SmithKline Beecham Animal Health (SBAH) vaccine strain ($BRSV^R$, SmithKline Beecham Animal Health Laboratories, Lincoln, NE) was propagated on bovine nasal turbinate (BT) cells (National Veterinary Service Laboratory, Ames, IA) maintained in Eagle's minimum essential media (EMEM);(GIBCO Bethesda Research Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS);(Hyclone, Logan, Utah), gentamicin sulfate (25 μg/ml);(SIGMA Immunochemicals, St Louis, MO), sodium penicillin G (25 μg/ml);(GIBCO), streptomycin sulfate (50 μg/ml);(GIBCO), neomycin sulfate (100 μg/ml);(GIBCO), and amphotericin B (25 μg/ml);(GIBCO). Cell cultures were incubated at 37°C in 5% CO_2.

Virus Purification

Extracellular virions were purified from two sources, tissue culture media recovered from BRSV-infected BT cells and reconstituted SBAH $BRSV^R$ vaccine. For harvesting virions from BRSV-infected BT cells, tissue culture roller bottles (490 cm^2);(Corning, Corning, NY) were inoculated with the SBAH $BRSV^R$ vaccine strain (one 25 dose vaccine vial per roller bottle) when BT cells reached a confluent monolayer. At

12 hours (hrs) PI, tissue culture media was aspirated and replaced with media containing 300μCi [^{3}H]uridine (NEN Dupont, Boston, MA) per 12ml media. The media was harvested at 24, 36, 48, and 60 hrs PI and replaced with media containing radiolabel as above.

For extracellular virions were purified from SBAH BRSV^R vaccine, vials of vaccine were reconstituted with half the recommended amount of diluent. Extracellular virions were purified from reconstituted vaccine by precipitation with polyethylene glycol (PEG), followed by banding in discontinuous and continuous sucrose gradients (Ueba 78).

The following procedures were carried out at 4°C. Harvested tissue culture fluid or reconstituted vaccine was centrifuged at 5,000 x g for 20 minutes to remove large cellular debris. A 50% PEG 8,000 (Sigma) solution in NT buffer (0.15 M NaCl, 0.05 M Tris-hydroxymethylaminomethane-chloride, pH=7.5) was added to the supernatant to a final concentration of 10%. The PEG solution was stirred for 60 minutes and centrifuged again at 5,000 x g for 10 minutes. The PEG-BRSV virion sediment (PEG pellet) was resuspended in 20% sucrose-NT buffer at 1/24th of the original material with a dounce homogenizer and centrifuged through a 30% sucrose-NT solution at 51,000 x g for 60 minutes. The 30% sucrose sediment (30% pellet) was resuspended in a small amount of 20% sucrose-NT buffer, layered on a 35-45-60% discontinuous sucrose-NT gradient, and centrifuged at 165,000 x g for 60 minutes. The distinct band, seen at the interface of the 35-45% sucrose boundary (discontinuous layer), was collected, layered on a linear 30-60% linear sucrose-NT gradient (linear gradient), and centrifuged at

165,000 x g for 180 minutes. The viral band, formed in the 1.18-1.22 g/cm^3 density region, was collected by fractionation of the entire gradient (5mls) into 25 separate aliquots.

Several control measures were performed to determine whether collected fractions contained virions. For extracellular virions purified from BRSV-infected BT cell tissue culture media, 100μl of the [^{3}H] radiolabeled material from the PEG pellet, the 30% pellet, the discontinuous layer, and each fraction of the linear gradient were placed in toluene-based scintillation fluid (New England Nuclear, Boston, MA). The [^{3}H] radioactivity was determined in counts per minute (cpm) by a scintillation reader (Beckman LS600TA, Arlington Heights, Il). For extracellular virions purified from reconstituted SBAH BRSV^R vaccine, the discontinuous gradient layer at the 35-45% sucrose interface and each fraction of the linear gradient were titrated for infectivity by plaque assay. Protein concentration was determined on fractions showing high viral titers (Bio-Rad Protein Assay, Bio-Rad, Richmond, CA). In addition, the density of the viral band at the linear gradient was determined in a sugar refractometer (Atago Optical Works, Japan);(Pringle 85).

Plaque Assay

The plaque assay for titration of virus infectivity was performed according to standard procedures (Kisch 63, Pringle 85) with the following modifications. Bovine turbinate cells were passaged onto 60mm diameter grided tissue culture plates (Corning) and allowed to reach a monolayer. Serial ten-fold dilutions of virus were made in EMEM containing horse serum (EMEM/HS);(Hyclone, Logan, Ut). Each dilution was

replicated three times. Media was removed from each plate and replaced with 100μl EMEM/HS and 100μl of the appropriate virus dilution. Virus was allowed to adsorb one hr at room temperature. Following adsorption, plates were overlaid with 4ml of 0.9% agarose (FMC Bioproducts, Rockland, ME) in EMEM supplemented with FCS and antibiotics as described under "virus and cells". To prevent plates from drying during incubation, 1ml of EMEM with FCS and antibiotics (as above) was added after the agar had solidified. Plates were incubated at 37°C for five days, examined, and plaques were counted at the highest dilution producing less than 100 countable plaques per plate.

Immunization

Six groups of four female Balb-C mice (six to eight weeks old);(Charles River, Portage, MI) were inoculated for immunization studies. One group of mice was inoculated with virus purified on the linear gradient (VL). For the initial inoculation, a dose of 1 x 10^5 PFU/mouse (2μg protein/mouse) was given by the intraperitoneal (IP) route in Freund's complete adjuvant (SIGMA). Mice were boostered twice at two week intervals and then monthly at the same dose and route, but incorporating Freund's incomplete adjuvant (SIGMA) until a fusion was performed. A second group of mice was inoculated in the same manner, but with virus recovered from the 35-45% sucrose interface of the discontinuous sucrose gradient (DISC).

A third group of mice was inoculated with the SBAH BRSV^R vaccine strain (BRSV^R). Vaccine was reconstituted with half the recommended amount of diluent, and 0.5 ml was given by the IP route (no adjuvant) following the immunization schedule described.

A fourth group of mice was inoculated with a killed whole cell culture (WCC) of BRSV-infected BT cells which was prepared following previously described procedures (Klucas 88). Briefly, BT cells were infected with the SBAH BRSV^R vaccine strain and harvested at 36-hrs PI (when cytopathic effect involved 50% of the monolayer) with 0.1% EDTA in phosphate buffered saline (PBS);(SIGMA). Harvested cells were suspended in PBS, centrifuged at 2,000 x g for ten minutes, washed twice in PBS, and fixed with 0.4% glutaraldehyde. Mice received approximately $1x10^7$ cells in 0.5 ml of PBS administered IP without adjuvant following our previously described immunization schedule.

The fifth and sixth group of mice were inoculated with lentil (*lens culinaris*) lectin coated agarose beads (Sigma) incubated with either tissue culture media harvested from BRSV-infected BT cell tissue cultures (BM) or from BRSV-infected BT cell lysates (BL). For the BM inoculum per mouse, BT cell monolayers in 150-cm^2 tissue culture flasks were infected with the SBAH BRSV^R vaccine strain at a multiplicity of infection of 0.3 and incubated for 36 hrs as above. Tissue culture media was then removed and centrifuged at 450 x g for 15 minutes at 4°C. A salt solution (0.1M NaCl, 0.1M $CaCl_2$, 0.1M $MnCL_2$) was added to the supernatant so that the final concentration was 0.1%. Then, 0.5ml of lectin coated agarose beads was added to the solution. After incubation at 4°C for 12 hrs with continuous shaking, the mixture was centrifuged as above, and the pellet washed three times with sterile PBS and mixed with 0.5ml PBS for injection. For the BL inoculum per mouse, BT monolayers were infected and incubated as above. After 36 hrs, infected flasks were placed on ice, washed three times with PBS,

supplemented with 12ml ice cold detergent buffer (0.01M NaH_2PO_4-Na_2HPO_4, 0.1 M NaCl, 1% Triton X-100, 0.5% NaDeoxycholate, 0.1% sodium dodecyl sulfate, pH=7.5) per 150-cm^2 flask, and incubated for five minutes. Lysates were collected by scrapping with a rubber policeman, centrifuged at 450 x g for 15 minutes, incubated with lectin coated agarose beads, and prepared for injection as above.

Production of Monoclonal Antibodies

Two lines of myeloma cells were used: NS-1 and SP2/0 (American Type Culture Collection, Rockville, Md). All procedures utilizing NS-1 cells were conducted in Dulbecco's Modified Eagle's Medium (DMEM);(SIGMA) containing FCS. All procedures utilizing SP2/0 cells were conducted in DMEM containing HS (Hyclone, Logan, Ut).

Mice used for fusion were immunized with the SBAH $BRSV^R$ vaccine strain of BRSV purified on the linear gradient. Three days prior to fusion, mice were boostered with four times the immunizing dose (no adjuvant): 1/2 given by the intravenous (IV) route utilizing the dorsal tail vein, 1/2 given by the IP route.

Fusion and establishment of hybridoma lines followed standard procedures (Harlow 88, Orvell 82, Sheshberadaran 85). Briefly, mice were sacrificed by CO_2 asphyxiation, and the spleen was aseptically removed and placed in 5ml of DMEM supplemented with 2.5% FCS (2.5% FCS/DMEM) with antibiotics as described above (under virus and cells). After teasing apart splenic tissue with forceps, the spleen cell suspension was collected and centrifuged at 450 x g for eight minutes. The supernatant was discarded, and 5ml of lysis buffer (8.29 g NH_4CL, 1.0 g $KHCO_3$, 0.037 g EDTA

in 1 liter distilled deionized water) was added for two minutes to lyse red blood cells. Then, 10ml of 2.5% FCS/DMEM was added, cells were centrifuged (450 x g for 8 minutes), and the supernatant was discarded. Another 10ml of 2.5% FCS/DMEM was added and cells were counted. Spleen cells ($1x10^8$ cells) were combined with myeloma cells ($1x10^7$ cells), centrifuged at 450 x g for eight minutes, and the supernatant was discarded.

For the fusion, 50% PEG (Boerhringer Mannheim, Indianapolis, IN) was used in the following manner: 800μl was added drop by drop over 1 minute, cells were gently stirred for two minutes, and 10ml of 2.5% FCS/DMEM was slowly added over a six minute period. After allowing cells to stand for two minutes, an additional 10ml of 2.5% FCS/DMEM was added slowly, cells were centrifuged (450 x g for 8 minutes), and the supernatant was discarded. Pelleted cells were resuspended in 10ml of 20% FCS/DMEM and allowed to stand for 30 minutes. Enough 20% FCS/DMEM was added to distribute cells to each of the inner wells (350μl/well) of sterile 96-well microtiter plates (Corning). Media (DMEM) without serum or antibiotics was added to the outer wells to provide a vapor barrier.

Twenty-four hrs later, 125μl of media was removed from each of the inner wells and replaced with 20% FCS/DMEM containing hypoxanthine (0.00136%)/aminopterin (0.00045%)/thymidine (0.00039%);(HAT);(SIGMA). Media was changed every three days. After 7 to 14 days, the HAT media was replaced with HT media (media as above without aminopterin). Fusion efficiency was determined on day seven by dividing the number of wells with at least one colony by the total number of wells seeded.

Hybridomas were expanded in 24-well plates (Corning), 25-cm^2 flasks (Fischer), and
cm^2 flasks (Fisher) as necessary.

During the first fusion, hybridomas producing antibodies specific to BRSV w
detected by two screening methods: enzyme-linked immunoassay (EIA) and indir
fluorescent antibody staining (IFA). Screening was performed at the level of 24-w
plates, 25-cm^2 flasks, and 75-cm^2 flasks. During subsequent fusions screening was do
by IFA only at the 96-well, 24-well, and 75-cm^2 flask level.

Positive wells were cloned twice by limiting dilution to ensure that antibo
producing cells were truly monoclonal and that the secretion of antibody could
maintained. Briefly, a 1:10 dilution of cells was made in HT media, and cell cou
were performed. A limiting dilution was then made such that one cell per well w
transferred to 96-well plates. Testing was repeated, and a second cloning by limit
dilution was performed.

Indirect Fluorescent Antibody Staining (IFA)

Bovine turbinate cells were infected with the SBAH $BRSV^R$ vaccine strain. Wh
viral cytopathic effect (CPE) involved approximately 50% of the monolayer, cells w
harvested in 0.5% trypsin-EDTA, washed twice in PBS, counted, and approximat
25,000 cells were spotted onto wells of teflon coated slides (Cell-Line Associat
Newfield, NJ). Slides were air dried at room temperature, fixed in acetone for
minutes, and stored at -70°C. Control slides were prepared similarly using uninfec
BT cells.

The IFA test was performed by covering the wells of infected and control sli

with 50μl of hybridoma media or ascites fluid, and incubating the slides for 30 minu at 37°C in a humidified chamber. Slides were washed in PBS, allowed to air dry, a 50μl of goat anti-mouse polyvalent immunoglobulin fluorescein isothiocyanate conjug (specific to IgA, IgM, IgG);(Sigma) was applied. A second incubation and washing w done. Slides were then counterstained with Evans Blue, air dried, mounted in glycer and examined by fluorescent microscopy. The F Mab to BRSV was used as a posit control, and PBS was used as a negative control.

Enzyme-linked Immunoassay (EIA)

The SBAH BRSV^R vaccine strain of BRSV was grown on BT cells in tis culture flasks and harvested when approximately 50% of the cell monolayer demonstra a CPE. Infected cells were removed from the flask in 0.5% trypsin-EDTA, wash twice in PBS, and dispensed into flat bottom 96-well polystyrene microtiter pla (approximately 50,000 cells per well). The plates were dried at room temperatu (23°C), blocked with 1% bovine serum albumin (Sigma) in tris-buffered saline (20m Tris hydroxymethylaminomethane, 500mM NaCl), and stored at 4°C until use.

The EIA was carried out as previously described (Engvall 72). Briefly, 50μl undiluted hybridoma culture fluid or mouse ascites fluid (diluted 1:20 in PBS) was add to wells and allowed to react at room temperature for one hr. The plates were wash three times in 0.05% Tween-20 tris-buffered saline, and 50μl of a 1:1000 dilution of g antibody to mouse immunoglobulin G conjugated to alkaline phosphatase (BioR Richmond, CA) was added. The plates were incubated at room temperature for one After washing as above, 50μl of 5 X diethanolamine substrate was added and plates w

incubated at room temperature for one hr. Optical density was read on an automated EIA reader (BioTech 312, Bio-Tek Instruments, Winooski, VT) with a 405 nm interference filter.

An F Mab against BRSV (obtained from Gary Anderson, University of Nebraska) was used as a positive control. Uninfected BT cells treated with all reagents except Mabs were used as negative controls. Positive wells were read as two to three times background. An absorbance greater than 0.2 was considered positive.

Concentration of Hybridoma Media

Hybridoma tissue culture media was collected and pooled by clone (or subclone) number. The media was then concentrated approximately 5-15 times based on volume using one of two methods. In one method, pooled media was passed through a positive-pressure N_2 ultrafiltration system (Amicon, Beverly, MA). The filter has a molecular weight cutoff of 50 kDa. In a second method, pooled media was passed through a 30 kDa filter by centrifugation at 1500 x g for 30 minutes (Amicon).

Preparation of Ascites Fluid

Mice were injected by the IP route with 0.5ml pristane (Sigma) seven days prior to injection of hybridoma cells. Approximately 1 x 10^6 hybridoma cells were washed twice and resuspended in sterile PBS. Then, 0.5ml was injected into 8 to 12 week old female Balb/C mice by the IP route. Ascites fluid was harvested in 14 to 21 days, when the abdomen of the mouse showed obvious fluid distension.

Radioactive Labeling of BRSV Viral Proteins

Standard methods for radioactive labeling were followed (Isfort 86). Uninfec control cells and cells infected with the SBAH BRSV^R vaccine strain at a multiplicity infection of 0.3 were labeled at 36 hrs PI. Monolayers in 150-cm^2 tissue culture fla were washed three times in Hanks balanced salt solution (GIBCO) and incubated fo hrs in 5ml of DMEM containing 1/20th of the normal concentration of methionine a 300μCi of [^{35}S]methionine (ICN-Flow) or 1/20th of the normal glucose concentration a 300μCi of [^{3}H]glucosamine (NEN Research Products, Boston, Mass).

After the 4 hr labeling, monolayers were washed three times with ice cold PB Then, 5ml of ice-cold detergent buffer (as above with 300μg of phenlymethylsulfor fluoride per ml and 0.23 trypsin-inhibiting units of aprotinin per ml);(Sigma) was adde and monolayers were incubated for five minutes at 4°C on ice (Whitte 79). Cells we then removed by scraping with a rubber policeman. The lysates were clarified centrifugation at 490 x g for 10 minutes at 4°C and stored at -20°C until used.

Radioimmunoprecipitation Assay (RIPA)

Bovine antisera produced to the 375 isolate of BRSV (provided by JC Bake Michigan State University, E. Lansing, MI) was obtained from a colostrum deprived c infected with the 375 BRSV isolate. The 375 isolate of BRSV is the origin of the SBA BRSV^R vaccine strain. The RIPA was performed using fetal bovine serum (Sigma) a mouse serum (Sigma) that were free of anti-BRSV antibodies.

Immunoprecipitation was performed by the methods of Witte and Wirth (7 Either 200μl of [^{35}S]methionine-labeled lysate or 600μl of [^{3}H]glucosamine-labeled lys

was first cleared with 1/20th of the total amount bovine serum free of antibody to BRSV and 1/20th of the total amount mouse serum free of antibody to BRSV. After incubation overnight at 4°C on ice, 10μl of a 10% *Staphylococcus aureus* protein-A preparation was added per 1μl of immune sera, and the mixture was incubated at 4°C on ice for one hr (Kessler 75). After incubation, the material was centrifuged at 1000 x g for three minutes. Approximately, 20μl of hybridoma or ascites fluid was added to the supernatant. Immune bovine antiserum and the BRSV F Mab were used as controls. After a second overnight incubation at 4°C on ice, a 10% *S. aureus* protein-A preparation was again added and incubated as above. The antigen-antibody-*S. aureus* protein-A complexes were harvested by centrifugation as above, washed three times in ice-cold detergent buffer (0.01M NaH_2PO_4-Na_2HPO_4, 0.1 M NaCl, 1% Triton X-100, 0.5% NaDeoxycholate, 0.1% SDS, pH=7.5), and collected by centrifugation at 1000 x g for three minutes. The pellet was suspended in sample buffer, heated at 68°C for 20 minutes, and centrifuged at 1000 x g for three minutes prior to electrophoresis.

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Discontinuous stack SDS-PAGE was performed according to the methods of Laemmli (70) in a vertical slab gel apparatus (Model SE 500, Hoeffer Scientific, Instruments, San Francisco, CA). A 12.5% polyacrylamide resolving gel was utilized for electrophoresis. Electrophoresis was carried out at a constant voltage of 150 V (Fisher Electrophoresis Power Supply model FB600, Fisher Scientific, Pittsburgh, PA). Gels were removed from the apparatus, fixed in glacial acetic acid (10% glacial acetic acid, 10% methanol);(EM Science, Gibbstown, NJ) overnight, and incubated in DMSO

(Sigma) twice for 30 minutes and in 26.7% PPO (J.T. Baker Inc., Phillipsburg, NJ) in DMSO for three hrs. Gels were dried for two hrs on an electrophoresis gel dryer and autoradiography of the dried gels was carried out at -70°C. Labeled proteins and glycoproteins were detected by fluorography as described by Bonner and Laskey (74). Standard molecular weight markers included myosin (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), B-lactoglobulin (18.3 kDa), and lysozyme (14.3 kDa);(GIBCO).

Virus Neutralization Assay

The capacity of Mabs to neutralize viral infectivity was determined by endpoint analysis. Serial two-fold dilutions of heat inactivated (56°C for 30 minutes) hybridoma fluid or ascites fluid were made in 96-well microtiter plates. One hundred PFU of the SBAH BRSV^R vaccine strain was added, and the virus-Mab mixture was incubated at room temperature for 1 hr. Then, BT cells were added to each well at a concentration that yielded a monolayer in 1 hr. After a five day incubation, the endpoint was determined as the highest dilution that completely inhibited syncytia formation. Complement enhanced neutralization was performed in parallel by the inclusion of a 1:24 dilution of fresh rabbit serum in the antibody mixture. Control experiments were done with heat inactivated rabbit serum.

Fusion Inhibition Assay

The ability to inhibit virus-induced cell fusion was determined by infection of BT cell monolayers in 96 well microtiter plates at a 0.5 multiplicity of infection of the SBAH

BRSV^{R} vaccine strain. Plates were incubated for 2 hrs at 37°C in 5% CO_2. The inoculum was discarded and serial two-fold dilutions of heat inactivated ascites fluid were added. Five days later the wells were inspected for syncytia formation. The endpoint was determined as the highest dilution of Mab that completely inhibited syncytia formation. Fusion inhibition studies were performed using ascites fluid.

Isotype

A commercial kit was used to determine isotype. The Immunoselect™ kit (GIBCO) utilized EIA for determining isotype specificity. The EIA was performed as described above, using ascites fluid diluted 1:20 in PBS. Isotyping was performed using ascites fluid.

RESULTS

Virus Purification

Extracellular virions were purified from tissue culture media harvested from BRSV-infected BT cells. [^{3}H]uridine was incorporated into viral RNA at various times PI, and the presence of extracellular virions in linear gradient fractions (#1-#25) was determined by measuring [^{3}H]uridine radioactivity in counts per minute (cpm). Counts per minute were highest in linear fractions #9 through #14 (total of 25 fractions) at 36, 48, and 60 hrs PI, with a peak in fractions #10 and #11 (Figure 1). Total cpm for all fractions increased from 24 to 60 hrs PI, with the greatest cpm occurring at 60 hrs PI. Total cpm decreased at each step during purification: the PEG pellet, the 30% pellet, the discontinuous layer, and the linear gradient (Table 4).

Extracellular virions were also purified from reconstituted SBAH BRSV^R vaccine. Titration of viral infectivity was determined by plaque assay for virions recovered from the 35-45% sucrose interface of the discontinuous sucrose gradient and from each fraction of the linear sucrose gradient. Titers of virions recovered from the discontinuous gradient ranged from 7-8 x 10^8 PFU/ml (data not shown). Titers of virions recovered from linear gradient fractions #9 through #14 (total of 25 fractions) ranged from 1x10^5 to 1x10^8 PFU/ml, with a peak in viral infectivity in fractions #10 and #11. Titers of virions recovered from other fractions of the linear gradient were negligible.

Response to Immunization

To determine if immunized mice responded with antibodies specific to BRSV proteins, serum from each mouse of all six groups ($BRSV^R$, DISC, VL, WCC, BM, BL) was analyzed by RIPA SDS-PAGE. Based on analysis of [^{35}S]methionine-labeled BRSV-infected BT cell lysates, a specific antibody response was detected to F_1, N, M, and P viral proteins in all groups of mice except the BM group (Figure 2). The BM mice responded to the F_1 and N protein, but did not respond to the P protein. A specific antibody response to membrane gps could be seen in each group of mice tested using [^{3}H]glucosamine-labeled BRSV-infected BT cell lysates. The $BRSV^R$, DISC, and VL groups of mice responded to the G, F_0, F_1, and F_2 gps (Figure 3A and 3B). The BM group of mice responded to the F_1 gp; however, no response to the G gp was noted in either the BL or the BM group. A [^{3}H]glucosamine-labeled BRSV-infected BT cell lysate RIPA SDS-PAGE was not done for the WCC group.

Results of Fusion and Screening

Fusions

Three fusions were performed using mice immunized with extracellular virions obtained from the SBAH $BRSV^R$ vaccine purified on the linear gradient. Fusion #1 was performed using NS-1 myeloma cells. Fusion efficiency, determined on day seven, was 58.5%. Screening was done at the 96-well microtiter plate, 24-well tissue culture plate, 25-cm^2 tissue culture flask, and 75-cm^2 tissue culture flask level by EIA and by IFA. Thirty-one hybridomas tested positive to BRSV-infected BT cells. Nine hybridomas were negative to BRSV-infected BT cells by IFA, but positive by EIA. Only those

hybridomas that were positive to BRSV-infected cells and negative to uninfected BT cells by IFA and EIA were subcloned, with one exception. Hybridoma 1F11, which was positive to BRSV-infected and uninfected BT cell by EIA and IFA, was subcloned.

A total of five hybridomas (1E7, 1F11, 2B3, 3G7, and 6E6) were subcloned twice by limiting dilution. All subclones were screened by IFA only. Subclones to 1E7 and 6E6 remained strongly positive to BRSV-infected BT cells and negative to uninfected BT cells by IFA. Subclones of 2B3 became weakly positive to BRSV-infected BT cells by IFA, but remained negative to uninfected BT cells. All subclones to 1F11 remained positive to BRSV-infected and uninfected BT cells by IFA except one (1F113E5), which was positive to BRSV-infected cells but negative to uninfected BT cells by IFA. Subclones of 3G7 became positive to both BRSV-infected and uninfected BT cells by IFA.

Fusion #2 was performed using SP2/0 myeloma cells and resulted in a 63% fusion efficiency. Hybridomas were screened at the 96-well microtiter plate and 75-cm^2 tissue culture flask level by IFA only. Hybridomas were tested by EIA at the 75-cm^2 tissue culture flask level. Twenty-five hybridomas were positive at the 96-well level. Of those 25 hybridomas, 17 remained positive to BRSV-infected BT cells by IFA during expansion through 25-cm^2 and 75-cm^2 tissue culture flasks. Of those 17 clones, 11 were positive to BRSV-infected cells and negative to uninfected BT cells by IFA, and 6 were positive to both BRSV-infected and uninfected BT cells. Only those hybridomas that were strongly positive to BRSV-infected cells and negative to uninfected BT cells by IFA were subcloned (8B2, 9E3, 10G3, 12B2, 12D7, and 12D10). All subclones remained

positive to BRSV-infected BT cells and negative to BRSV-uninfected BT cells by IFA.

Fusion #3 was also performed using SP2/0 myeloma cells. Although fusion efficiency was 37%, no hybridomas were positive to BRSV-infected BT cells by IFA.

Immunofluorescent Staining

Different IFA staining patterns were observed for various hybridomas, and Mabs could be grouped according to staining pattern. Group I antibodies produced an immunofluorescent pattern on the perimeter of acetone fixed cells (Figure 4). The majority of stained cells exhibited a granulated annular fluorescent pattern. Group I hybridomas included 1E72C4, 1F3, 2B31D4, 3G71D4, 8B21E7, 10G31B2, 11C7, 11G4, 12B21C6, and 12D7.

The immunofluorescence pattern of Group II antibodies differed from Group I antibodies in that the Group II immunofluorescence pattern was less granular than Group I and delicately stained intracytoplasmic areas. Group II hybridomas included 1F113E5, 6D4, 6E62B8, 9D11, 9E31E5, 9E11, 12D101F2, 13C5, 13C8, and 13G5.

Enzyme-linked Immunoassay (EIA)

Although the EIA was performed using standard techniques and equipment, extensive background absorbance occurred during hybridoma screening. Blocking the EIA plates with 1% bovine serum albumin in tris-buffered saline did not prevent nonspecific protein binding.

Characterization of Hybridomas

RIPA and SDS-PAGE

Twenty hybridomas were analyzed by RIPA SDS-PAGE using [^{35}S]methionine- and [^{3}H]glucosamine-labeled BRSV-infected BT cell lysates (Table 3). Radiolabeled mock infected BT cells were used as controls.

Of the 20 hybridomas tested by RIPA SDS-PAGE, 19 immunoprecipitated at least one or more viral proteins. One Mab, 1E72C4, immunoprecipitated the F_0 gp based on [^{3}H]glucosamine-labeled BRSV-infected BT cell lysate (Figure 6); however, it also immunoprecipitated the N protein based [^{35}S]methionine-labeled lysate. One Mab, 8B21E7, immunoprecipitated the F_0, F_1, and F_2 gps based on [3H]glucosamine-labeled BRSV-infected BT cell lysate (Figure 7), but also immunoprecipitated the N protein using [^{35}S]methionine-labeled lysate. Using [35S]methionine-labeled BRSV-infected BT cell lysate, eleven hybridomas (1F3, 2B31D4, 3G71D4, 9D11, 10G31B2, 11C7, 11G4, 12B21C6, 12D7, 13C8, 13G5) immunoprecipitated the F_1 and N gp (Figure 8). One hybridoma (12D101F2) immunoprecipitated N and P, and five hybridomas precipitated the N protein. No Mabs immunoprecipitated the G gp. Results of the RIPA SDS-PAGE analysis using [^{35}S]methionine labeled lysate were the same using concentrated tissue culture hybridoma fluid or ascites fluid.

Virus Neutralization

Of the twenty hybridomas tested only one hybridoma, 8B21E7, produced Mabs that neutralized virus in the absence of complement. Five hybridomas neutralized virus

to a dilution of 1:4 or 1:8 in the presence of complement. These five hybridomas immunoprecipitated the N protein with either the F_0 or F_1 gp. In the presence of complement, the neutralization titer of 8B21E7 did not change. Results of virus neutralization were the same using concentrated hybridoma fluid or ascites fluid.

Virus-induced Fusion Inhibition

One hybridoma (8B21E7) inhibited fusion to a titer of 1:32. This hybridoma immunoprecipitated the F_0, F_1, and F_2 gps based on [^{3}H]glucosamine-labeled BRSV-infected BT cell lysate in RIPA SDS-PAGE, and immunoprecipitated the N protein based on [^{35}S]methionine-labeled BRSV-infected BT cell lysate.

Enzyme-linked Immunoassay (EIA)

Four hybridomas were strongly positive by EIA, six hybridomas were weakly positive, and four hybridomas were negative (Table 3).

Isotype

Only hybridomas that were subcloned twice by limiting dilution were isotyped. Ten subclones were of the IgG_{2b} isotype. Eight of these subclones had kappa light chains, and two subclones had lambda light chains.

Table 3. Characterization of hybridomas.

	SDS-PAGE	Neutralization complement without	Neutralization complement with	Fusion Inhibition	Isotype heavy/light
Fusion #1					
1E72C4	N/F_0	-	1:8	-	IgG2b/κ
1F3	N/F_1	-	1:8	-	ND*
1F113E5	N	-	-	-	IgG2b/κ
2B31D4	N/F_1	-	1:8	-	IgG2b/λ
3G71D4	N/F_1	-	-	-	IgG2b/λ
6D4	N	-	-	-	ND*
6E62B8	N	-	-	-	IgG2b/κ
Fusion #2					
8B21E7	N/F	1:4	1:4	1:32	IgG2b/κ
9E31E5	N	-	-	-	IgG2b/κ
9D11	N/F_1	-	-	-	ND*
9E11	N	-	-	-	ND*
10G31B2	N/F_1	-	-	-	IgG2b/κ
11C7	N/F_1	-	-	-	ND*
11G4	N/F_1	-	-	-	ND*
12B21C6	N/F_1	-	-	-	IgG2b/κ
12D101F2	N/P	-	-	-	IgG2b/κ
12D7	N/F_1	-	1:4	-	ND*
13C5	-	-	-	-	ND*
13C8	N/F_1	-	-	-	ND*
13G5	N/F_1	-	-	-	ND*
Controls					
IBS		>1:2048	>1:2048	-	
F Mab		1:4	1:4	1:32	

IBS = Immune bovine serum to BRSV
*ND = Isotype was determined only for hybridomas subcloned twice by limit dilution
S/+ = Strong positive
W/+ = Weak positive
- = Negative

Table 4. [^{3}H]uridine activity in cpm in the PEG pellet, 30% sucrose pellet, 35-45% sucrose interface of discontinuous sucrose gradient, and in 25 fractions of the linear sucrose gradient of virions purified from BRSV-infected BT cells.

SOURCE	24 hr	36 hr	48 hr	60 hr
PEG Pellet	1,476	5,286	9,936	43,804
30% Pellet	386	1,870	2,778	10,056
Discontinuous layer	30	122	140	532
Linear Gradient				
1	32	26	24	31
2	20	10	40	26
3	14	10	16	20
4	31	37	36	26
5	16	32	16	32
6	20	34	15	58
7	16	31	24	53
8	16	24	65	91
9	26	49	86	163
10	16	75	102	500
11	31	120	92	480
12	31	115	118	380
13	26	99	131	345
14	42	64	85	188
15	35	42	46	106
16	18	34	32	47
17	20	27	18	80
18	17	37	29	46
19	25	30	29	36
20	16	23	22	26
21	25	34	30	55
22	16	25	22	35
23	20	20	20	30
24	15	15	15	18
25	10	10	18	15

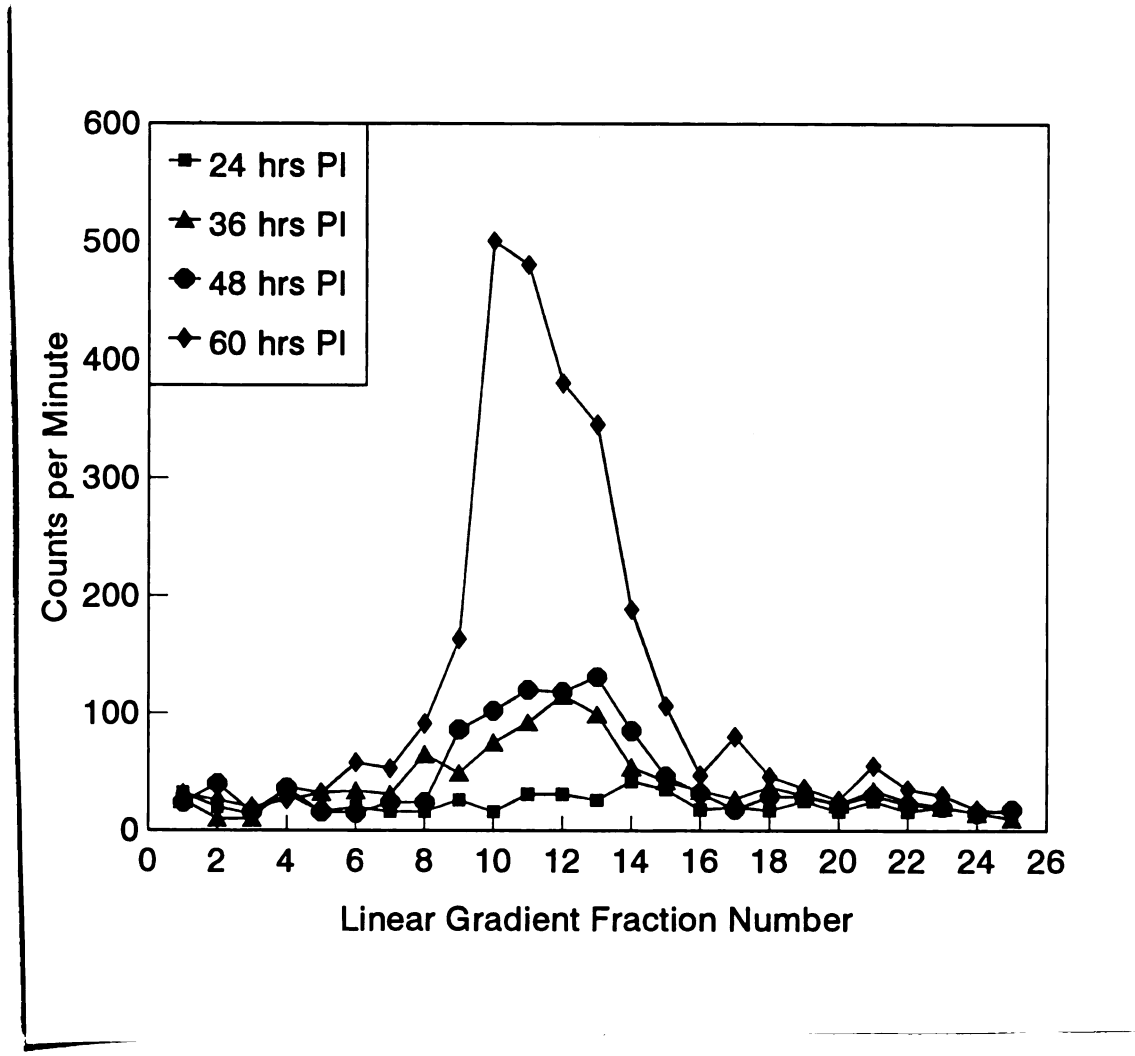

Figure 1. Graph of [^{3}H]uridine radioactivity in cpm in 25 fractions of the linear sucrose gradient. Media was harvested from BRSV-infected BT cells at 24, 36, 48, and 60 hrs PI.

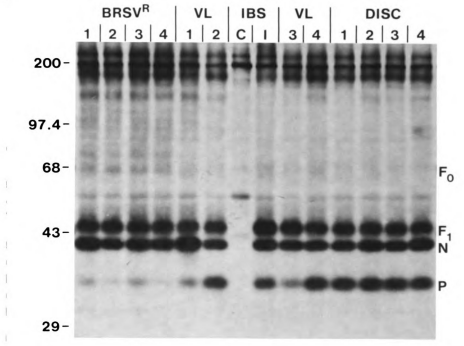

Figure 2. Results of immunization of mice. Immunoprecipitation and SDS-PAGE analysis of [^{35}S]methionine-labeled BRSV-infected BT cells. Labeling was for 4 hrs at 36 hrs PI. Immunoprecipitation was performed using mouse anti-BRSV polyclonal serum. BRSV^R indicates mice immunized with reconstituted SBAH BRSV^R vaccine, DISC indicates mice immunized with purified extracellular virions obtained from the 35-45% sucrose interface of the discontinuous sucrose gradient, and VL indicates mice immunized with extracellular virions purified on the linear sucrose gradient. IBS indicates immune bovine serum to BRSV (C=Control or mock infected BT cells, I=BRSV-infected BT cells). Mock infected BT cells were used as negative controls (data not shown).

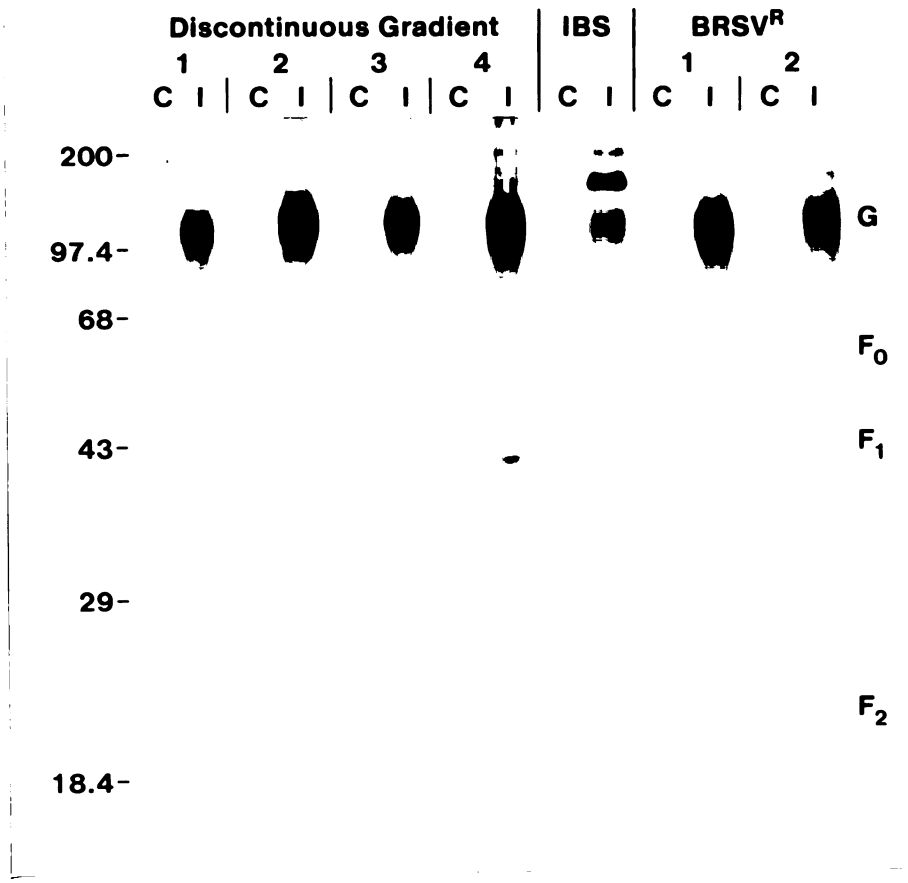

Figure 3A. Results of immunization of mice. Immunoprecipitation and SDS-PAGE analysis of [^{3}H]glucosamine-labeled BRSV-infected BT cells. Labeling was for 4 hrs at 36 hrs PI. Immunoprecipitation was performed using mouse anti-BRSV polyclonal serum. Discontinuous Gradient indicates mice immunized with purified virions obtained from the 35-45% interface of the discontinuous sucrose gradient, and BRSV^R indicates mice immunized with reconstituted SBAH BRSV^R vaccine. IBS indicates immune bovine serum to BRSV (C=Control or mock infected BT cells, I=BRSV-infected BT cells). Mock infected BT cells were used as negative controls.

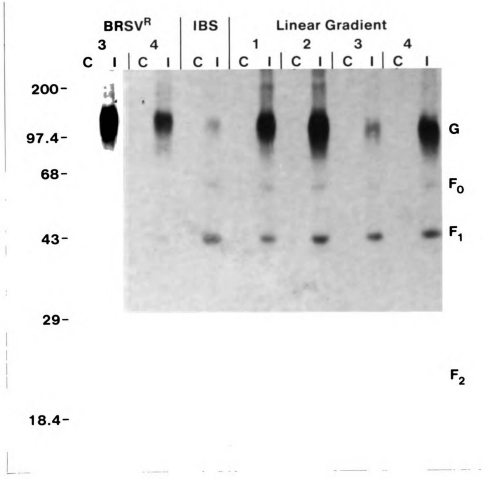

Figure 3B. Results of immunization of mice. Immunoprecipitation and SDS-PAGE analysis of [^{3}H]glucosamine-labeled BRSV-infected BT cells. Labeling was for 4 hrs at 36 hrs PI. Immunoprecipitation was performed using mouse anti-BRSV polyclonal serum. BRSV^R indicates mice immunized with reconstituted SBAH BRSV^R vaccine, and Linear Gradient indicates mice immunized with virions purified on the linear sucrose gradient. IBS indicates immune bovine serum to BRSV (C=Control or mock infected BT cells, I=BRSV-infected BT cells). Mock infected BT cells were used as negative controls.

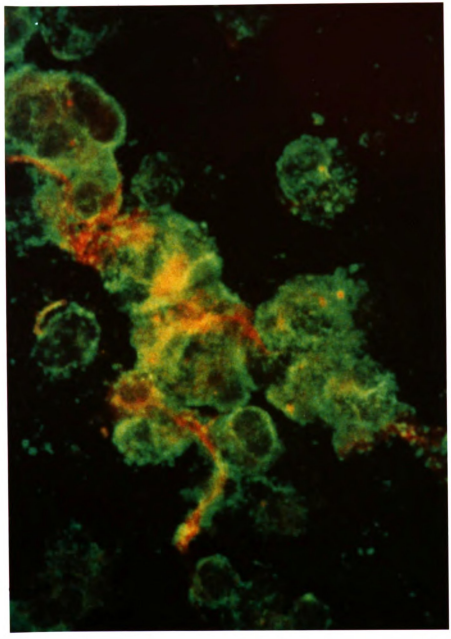

Figure 4. Photomicrograph of Group I type indirect immunofluorescent antibody staining of BRSV-infected BT cells (by hybridoma 1E72C4). The Group I type Mabs produced a granular immunofluorescent pattern that stained the outer portions of BRSV-infected BT cells. Photographs were taken at a magnification of x225.

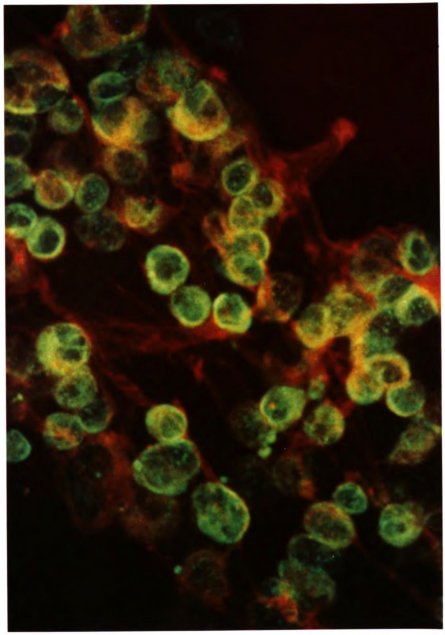

Figure 5. Photomicrograph of Group II type indirect immunofluorescent antibody staining of BRSV-infected BT cells (by hybridoma 9D4). The Group II type immunofluorescent pattern was less granular than Group I and stained intracytoplasmic areas. Photographs were taken at a magnification of x225.

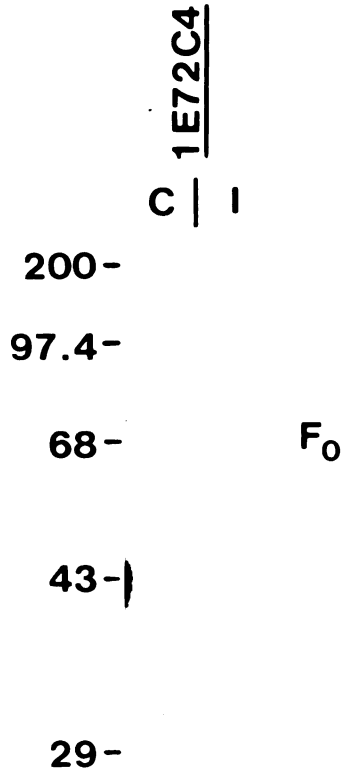

Figure 6. Characterization of hybridoma 1E72C4 by immunoprecipitation and SDS-PAGE analysis of [^{3}H]glucosamine-labeled BRSV-infected BT cells. Labeling was for 4 hrs at 36 hrs PI. Immunoprecipitation was performed using mouse anti-BRSV ascites fluid. Mock infected BT cells were used as negative controls (C=Control or mock infected BT cells, I=BRSV-infected BT cells).

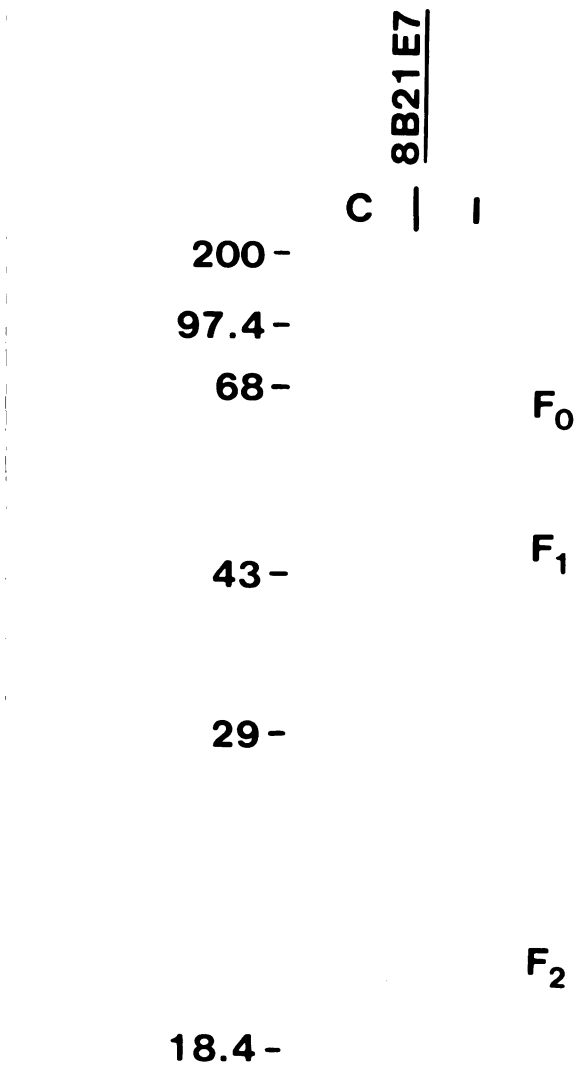

Figure 7. Characterization of hybridoma 8B21E7 by immunoprecipitation and SDS-PAGE analysis of [^{3}H]glucosamine-labeled BRSV-infected BT cells. Labeling was for 4 hrs at 36 hrs PI. Immunoprecipitation was performed using mouse anti-BRSV ascites fluid. Mock infected BT cells were used as negative controls (C=Control or mock infected BT cells, I=BRSV-infected BT cells).

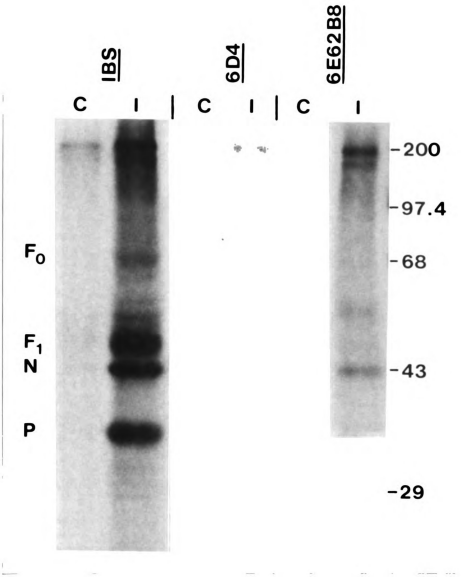

Figure 8. Characterization of hybridomas 6D4 and 6E62B8 by immunoprecipitation and SDS-PAGE analysis of [^{35}S]methionine-labeled BRSV-infected BT cells. Labeling was for 4 hrs at 36 hrs PI. Immunoprecipitation was performed using mouse anti-BRSV ascites fluid. IBS indicates immune bovine serum to BRSV (C=Control or mock infected BT cells, I=BRSV-infected BT cells). Mock infected BT cells were used as negative controls.

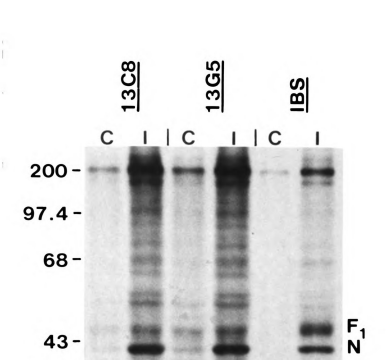

Figure 9. Characterization of hybridomas 13C8 and 13G5 by immunoprecipitation
SDS-PAGE analysis of [^{35}S]methionine-labeled BRSV-infected BT cells. Labeling
for 4 hrs at 36 hrs PI. Immunoprecipitation was performed using mouse anti-BR
ascites fluid. IBS indicates immune bovine serum to BRSV (C=Control or m
infected BT cells, I=BRSV-infected BT cells). Mock infected BT cells were used
negative controls.

DISCUSSION

Virus Purification

Because of the inherent lability and the difficulty in propagating RSV, there are few reports of HRSV purification and no reports of BRSV purification. Attempts to purify HRSV resulted in poor total yields with loss of infectivity because the virus tends to remain associated with the cellular membrane (Levine 77, Wunner 76). However, we were able to purify extracellular virions that retained infectivity from both BRSV-infected BT cells and from reconstituted SBAH BRSV^{R} vaccine in yields that were sufficient to immunize mice.

A [^{3}H]uridine radiolabel was used to monitor the presence of extracellular virions during purification of BRSV-infected BT cells. Based on cpm of [^{3}H]uridine radioactivity, more extracellular virions could be recovered from BRSV-infected BT cells at 60 hrs PI than at 24, 36, or 48 hrs PI. The higher cpm at 60 hrs PI may have been due to either virus budding or to excessive loss of lytic BRSV-infected BT cells into the media. Scintillation assays of the linear sucrose gradient fractions (final purification step) indicated the presence of extracellular virions in fractions #9 through #14 (25 total fractions). Since the principle of density-gradient centrifugation is the segregation of particles on the basis of their densities, material differing from the virus in density are

separated. The viral band in linear fractions #9-#14 formed in the 1.18-1.22 g/cm^3 density region, in accordance with previously published reports of RSV density (Pringle 85). These results demonstrate that BRSV can be purified from BRSV-infected BT cells. The ability to purify extracellular virions from tissue culture media harvested from BRSV-infected BT cells is important for future studies designed to produce Mabs against viral proteins of other BRSV isolates.

Extracellular virions were also purified from reconstituted SBAH BRSV^R vaccine. In cell culture systems, RSV typically exhibits two features that correspond to pathogenic functions, infectivity and syncytia formation (Cote 81). Infectivity is the only reliable marker available to follow the virus during nonradiolabeled purification procedures (Cote 81). Based on plaque assays, purified virions retained infectivity which was highest in linear fractions #9 through #14 (25 total fractions) where infectivity titers ranged from 1X10^5 to 1x10^8 PFU/ml. Retention of infectivity indicates that the virion envelope is intact and that purified virions are capable of attachment and cell-to-cell fusion, functions of the G and F gps, respectively. The location of viral infectivity in linear sucrose gradients of virions purified from SBAH BRSV^R vaccine as determined by plaque assay correlated well with the location of virions in linear sucrose gradients of virions purified from BRSV-infected BT cells as determined by scintillation assays of [^{3}H] radioactivity.

These results indicate that not only can BRSV be purified from BRSV-infected BT cells or from reconstituted SBAH BRSV^R vaccine, but also that purified BRSV can retain infectivity and that purified BRSV can be obtained in sufficient amounts (0.02μg/μl) to immunize mice for the production of Mabs. Purification of virions from reconstituted

SBAH BRSV^R vaccine is less time consuming, less costly, and easier to accomplish than purifying virions from BRSV-infected BT cell media. To produce panels of Mabs to isolates other than the SBAH BRSV^R vaccine strain, however, extracellular virions must be purified from tissue culture media harvested from BRSV-infected BT cells. The work reported here establishes that method for future use.

Response to Immunization

Several methods were developed to prepare BRSV antigen for the immunization of mice. Mice were inoculated with: extracellular virions purified on the 35-45% sucrose interface of the discontinuous sucrose gradient (DISC), extracellular virions purified on the linear sucrose gradient (VL), reconstituted SBAH BRSV^R vaccine (BRSV^R) administered directly to mice, glutaraldehyde fixed BRSV-infected whole BT cell cultures (WCC), and lentil lectin coated agarose beads incubated with either tissue culture media (BM) or lysates (BL) from BRSV-infected BT cells. Based on RIPA SDS-PAGE analysis of [^{35}S]methionine- and [^{3}H]glucosamine-labeled BRSV-infected BT cell lysate, a specific immune response to the G, F_1, F_2, N, and P proteins was produced in the BRSV^R, DISC, and VL groups of mice. The WCC group produced a specific immune response to F_1, N and P proteins (RIPA SDS-PAGE analysis using [^{3}H]glucosamine-labeled BRSV-infected BT cells was not done for the WCC group). These results demonstrate that when mice are immunized to BRSV, a specific antibody response to at least four structural proteins, most importantly to the G gp, can be obtained. Obtaining a Mab to the BRSV G gp is essential to the identification of BRSV subgroups since the majority of antigenic variation in BRSV, like HRSV, may lie in the

G gp. Because purified virions reduce the antibody response of the mouse to nonviral antigens (Campbell 84), extracellular virions purified from the linear gradient were used for all fusions.

Lectins are proteins that precipitate complex carbohydrates, especially diverse oligosaccharides of cell surface glycoproteins. Lentil lectin coated agarose beads should bind viral glycoproteins, and, in the case of BRSV, only the F and G gps. Lentil lectin coated agarose beads incubated with tissue culture media harvested from BRSV-infected BT cells (BM) should selectively bind the soluble form of G (Hendricks 87). The BM group of mice produced antibodies to the F_1 and N protein, but not the G gp. The lectin coated beads may have bound glycoproteins on extracellular virions that were shed into the media from BRSV-infected BT cells; therefore, mice immunized by the BM method would respond to several viral proteins. Pelleting extracellular virions by ultracentrifugation of the media or by precipitation with PEG prior to incubation with lectin coated beads may improve results of the BM immunization method.

Lentil lectin coated agarose beads incubated with lysates of BRSV-infected BT cells (BL) should selectively bind the F and G viral gps. The BL group responded to the F, N, and P proteins, but did not respond to the G gp. Mice immunized by the BL method produced antibodies to several viral proteins probably because the lectin coated beads bound glycoproteins on either intact virions or on vesicles containing viral proteins. Based on these results, using beads incubated with BRSV-infected BT cell lysates to select for viral gps was ineffective.

Several other immunization protocols could be examined that might result in a

Mab specific to the G gp after a fusion. First, column chromatography using Concanavalin A (a lectin) could be utilized to affinity purify the BRSV G gp which could then be used as an immunogen (Bernstein 80, Lambert 83, Walsh 84). One report indicated that almost 50% of a fusion protein applied to an immunoadsorbent column could be recovered in an antigenically active form (Stott 84b). Second, the BRSV G gp could be removed from RIPA SDS-PAGE gels, recovered by electroelution through a small membrane (10 kDa molecular weight cut off), and used as an immunogen. Alternatively, the purified G obtained from SDS-PAGE could be injected into rabbits to produce rabbit anti-G. The rabbit anti-G could then be used to purify BRSV G by immunoaffinity column chromatography. Third, since the complete nucleotide sequence of the BRSV G gene is known and since the BRSV G gene can be amplified using polymerase chain reaction technology and cloned into vectors (using vaccinia or adenovirus vectors or a bacculovirus or semiliki virus expression system), the BRSV G gp could be produced in large quantities and used as an immunogen. And fourth, immunization of mice with a viable Balb/c mouse cell line persistently infected with HRSV has proven successful in the production of monoclonals to the HRSV G gp (Fernie 81,82).

Screening of Hybridomas

Two methods were utilized to screen hybridomas for the production of Mabs specific to BRSV viral proteins, enzyme-linked immunoassay (EIA) and indirect immunofluorescence (IFA). Although the EIA worked well in preliminary experiments to identify the F Mab used as a control, similar results were not obtained using

hybridoma or ascites fluid during screening. A variable amount of background absorbance occurred in both uninfected and infected wells despite blocking for nonspecific protein binding.

Increased background absorbance may have been caused by nonspecific binding of proteins in the sample, from loss of antigen in test wells, or from the type of EIA plate used. Hybridoma fluid, especially when concentrated, can contain large amounts of protein contaminants. These contaminants are largely albumins, but may contain bovine immunoglobulins (if FCS is used during the fusion), proteases, and nucleases (Campbell 84). Ascitic fluid contaminants also include nonspecific immunoglobulins together with larger amounts of proteases and nucleases (Campbell 84).

Loss of antigen from test wells increases the likelihood of nonspecific protein binding to the EIA plate. Test wells were plated with either uninfected or BRSV-infected BT cells, allowed to dry at room temperature, and stored at -20°C until used. Antigen could have been lost from test wells during drying by desiccation, during storage by freeze-thaw fracture, or during EIA assays by washing. One way to avoid antigen loss would be to fix antigen to test wells with either ethanol or acetone (Bolin 91). Fixing antigen as above and hand washing during the EIA procedure may prevent antigen loss during the EIA procedure.

Alternatively, nonspecific protein binding may have been a result of the type of EIA plate or the type of tissue culture serum used. The EIA was developed using techniques similar to others who had successful results (Baker 92, Mufson 85). One difference, however, was the type of EIA plate utilized. Anecdotal reports abound

concerning the success of some types of EIA plates over others (Liddell 91). Another difference was the use of DMEM containing horse serum. To avoid problems in the future, the EIA should be tested prior to screening using a variety of plates and different lots of the tissue culture serum to be utilized during production of hybridomas.

In contrast to the EIA, the IFA worked well for screening hybridomas for Mabs specific to BRSV viral proteins. Two distinct patterns of immunofluorescent staining were observed. Group I hybridomas produced an annular staining pattern with a distinctly granular appearance. The staining pattern of the Group II hybridomas was less granular and stained intracytoplasmic areas as well as annular areas. The two group division was developed by researchers studying Mabs to HRSV (Cote 81, Fernie 82, Gimenez 84, Klucas 88, Stott 84b, Walsh 83a). These researchers determined that the Group I staining pattern was consistent with specificity of Mabs for a protein expressed on the cell surface, while the Group II staining pattern was consistent with hybridomas producing Mabs to proteins located in intracellular virions.

Because the IFA screening procedure was faster, less costly, and easier to perform than the EIA, screening was done by IFA only during the second and third fusion. One disadvantage of screening by one method only is that some monoclonals may fail to react in the screening method being used.

Why some Mabs fail to react by IFA or EIA is unknown. Similar results have been reported by others working on HRSV (Anderson 85, Gimenez 86, Routledge 85). Anderson, *et al* (85) found that different epitopes showed varying sensitivities to IFA acetone fixation and suggested that some epitopes may be lost during IFA preparation.

Some Mabs to the F gp may have a low affinity for antigen resulting in low EIA absorbance readings (Routledge 85). In addition, since hybridomas secrete varying amounts of antibody, low EIA absorbance for some Mabs is possible.

Only those hybridomas that were positive by EIA and IFA, and only those hybridomas that were positive to BRSV-infected BT cells and negative to uninfected BT cells by IFA, were subcloned and further characterized. Clones that were positive to both BRSV-infected and uninfected BT cells may contain a hybridoma producing Mabs specific to BRSV. In addition, those clones that were positive by EIA but negative by IFA may also contain hybridomas producing Mabs specific to BRSV.

Characterization of Hybridomas

The primary purpose of this project was to produce and characterize a panel of Mabs to the SBAH BRSV^R vaccine isolate (375) of BRSV. Only two previous reports of Mabs specific to BRSV exist (Kennedy 88, Klucas 88). Klucas, *et al* (88) produced five Mabs to the 375 isolate of BRSV, of which two were specific to the F gp, and three were not characterized as to protein specificity. The second report describes the production of six interspecies hybridomas (heterohybridomas using bovine lymphocytes and mouse myeloma cells) secreting Mabs against F_0, F_1, and F_2 gps of the 127 isolate of BRSV (Kennedy 88).

The current report describes the production and characterization of 19 hybridomas reactive with the F_0, F_1, F_2, N, and P viral proteins of the SBAH BRSV^R vaccine strain of BRSV (375 isolate). All hybridomas were of the IgG_{2b} subtype with either kappa or lambda light chains. One hybridoma (1E72C4) produced Mabs reactive with the F_0 gp

based on [^{3}H]glucosamine-labeled BT cell lysates in RIPA SDS-PAGE analysis, but also immunoprecipitated the N protein based on [^{35}S]methionine-labeled BRSV-infected BT cell lysates in RIPA SDS-PAGE analysis. One hybridoma (8B21E7) produced Mabs that reacted to the F_0, F_1, and F_2 gp, but also immunoprecipitated the N protein. Eleven hybridomas immunoprecipitated N and F_1 proteins, but did not immunoprecipitate a viral envelope gp. One hybridoma immunoprecipitated the N and P protein, and five hybridomas immunoprecipitated the N protein.

The RSV fusion protein (F_0) is a 68 kDa inactive precursor protein that is proteolytically cleaved into two active subunits, F_1 and F_2. The hybridoma producing Mabs to the F_0 gp (1E72C4) neutralized virus only in the presence of complement and did not inhibit fusion. The hybridoma producing Mabs reactive with the F gp (8B21E7), however, neutralized virus with and without complement and inhibited fusion. Many reports exist of Mabs specific to the HRSV F gp that neutralize virus with and without complement and inhibit fusion (Fernie 82, Olmstead 86, Orvell 87 Samson 86, Walsh 83a). These reports also describe Mabs to the F gp that neither neutralize virus nor inhibit fusion. The presence of complement has been reported to increase the neutralization titer four to five-fold (Buynak 79, Fernie 82, Tsutsumi 87) suggesting that neutralization is mediated by antibody dependent viral membrane lysis (Walsh 83a). Since the fusion protein is involved in the fusion of enveloped viruses to host cell membranes, neutralization of RSV by anti-F antibodies may be mediated by inhibition of virus-to-cell fusion.

One Mab, 8B21E7, gave an unusual pattern of reactivity when tested by

[^{3}H]glucosamine-labeled BRSV-infected BT cell lysate in RIPA SDS-PAGE in that it immunoprecipitated the F_0, F_1, and F_2 components of the F gp. Although a given Mab would be expected to react with a unique epitope within a protein, similar results of an HRSV anti-F mab reacting to both the F_1 and F_2 subunits have been reported (Garcia-Barreno 89, Mufson 85, Orvell 87, Samson 86, Trudel 86, Tsutsumi 87, Walsh 83a,86). Samson, *et al* (86) suggested that this unusual behavior may be due to: 1) biclonal hybridomas where one clone produces antibodies to F_1 while the other clone produces antibodies to F_2, 2) a 21 kDa portion of F_1 that is cleaved and carries the same epitope (and therefore mimics F_2 binding), or 3) a common epitope within F_1 and F_2. Since the 8B21E7 hybridoma was subcloned twice by limiting dilution, the biclonal theory seems unlikely. No reports of a 21 kDa fraction of F_1 exist, although further experiments may be needed to establish whether such a fragment could be derived from F_1. Samson, *et al* (86) proposed the term "geminiepitopic" to refer to the situation when a Mab reacts with two distinct epitopes occurring on the same protein molecule.

Many hybridomas immunoprecipitated the N protein either alone or with the P or F_1 protein. The RSV N protein is an abundant structural protein of the viral nucleocapsid that is tightly complexed with viral genomic RNA. There are several reports of HRSV Mabs to P coprecipitating with N (Gimenez 84, Mufson 85, Orvell 87, Routledge 85, Stott 84b). In addition, several reports exist of an HRSV anti-F Mab immunoprecipitationg both the F and N proteins (Mufson 85, Orvell 87). Gimenez, *et al* (84) considered it unlikely that N and P share common epitopes. Routledge, *et al* (85) suggested that N and P form a macromolecule since the band associated with the N

protein was greatly reduced when immunoprecipitation was repeated after ultracentrifugation (100,000 x g for 45 minutes) or after addition of the nonionic detergent Empigen in the RIPA buffer (Routledge 85). Orvell, *et al* (87) determined that antibodies against the P protein coprecipitated the N protein, but that antibodies against the N did not coprecipitate the P protein. Since some of the hybridomas that coprecipitated the N with either P or F_1 were not subcloned, it is possible that two hybridomas exist, one specific to N and one specific to either P or F_1.

Despite the fact that N is an abundant protein, it is somewhat surprising that so many hybridomas secreted antibodies to N. There are no reports of an HRSV anti-N Mab that neutralizes or inhibits fusion. Although it is possible that an anti-F Mab (immunoprecipitating the F and N proteins) neutralized virus or inhibited fusion, it is also possible that the highly concentrated hybridoma fluid or impure ascites fluid may have interfered with virus neutralization and fusion inhibition tests. Many standard protocols exist for purifying Mabs from ascites or tissue culture hybridoma fluid including ammonium sulfate precipitation, diethylaminoethyl-cellulose column dialysis, and affinity chromatography (Campbell 84). Perhaps characterization of Mabs by ability to neutralize virus in the presence of complement and ability to inhibit viral-induced fusion should be repeated using purified Mabs.

The RIPA SDS-PAGE analysis did not enable the specificity of all Mabs to be definitively identified. First, some EIA and IFA positive Mabs failed to react in RIPA SDS-PAGE. Similar results have been reported with Mabs to HRSV (Fernie 82, Storch 87, Stott 84b). Fernie, *et al* (82) suggested that failure to immunoprecipitate viral

proteins may be due to SDS-PAGE alteration of protein conformation and classi
Mabs by radioimmunoassay (Fernie 82). Stott, *et al* (84b) used competitive bin
assays to identify Mabs that failed to react by RIPA SDS-PAGE. Monoclonal antit
specificity could be also examined by western immunoblot assay (Stott 84). Howe
Klucas, *et al* (88) found that not all BRSV Mabs reacted by Western immunot
Alternatively, if viral proteins could be purified, then Mabs could be identified by
using the purified protein as the test antigen or by competitive blocking studies u
SDS-PAGE.

Second, analysis of RIPA SDS-PAGE autoradiographs indicated the presenc
unidentified nonspecific polypeptides. The presence of nonspecific protein bindin
RIPA SDS-PAGE suggests either immunoprecipitation of nonspecific proteins or c
contamination of infected to control lanes. Addition of mouse serum (free of antibo
to BRSV) during the preclear step of the immunoprecipitation did not elimi
nonspecific protein binding. One problem may have been the use of impure ascites f
or concentrated tissue culture hybridoma media. Similar nonspecific protein binding
occurred with other researchers. Stott, *et al* (84) suggested that the "contamination"
be due to "the difficulty in disrupting membrane bound vesicles" or to the possibility
some of the proteins precipitated may be precursors to or degradation products of o
viral polypeptides. Walpita, *et al* (92) found that viral proteins were obscured
numerous host-cell specific protein bands produced during one-dimensional SDS-PA
separation of virus-infected whole-cell lysates. In addition, Mabs to viral proteins
cross-react with host proteins (Tsutsumi 87). One study suggested that nearly 3.5%

all Mabs produced to other viruses cross-react with host proteins (Srinivasappa 86).

Third, and most importantly, the [^{3}H]glucosamine-labeled RIPA SDS-PAGE never consistently allowed identification of viral surface membrane gps. Analysis of immunized mice sera by [^{3}H]glucosamine-labeled BRSV-infected BT cell lysate RIPA SDS-PAGE indicated that all surface glycoproteins, including G, F_0, F_1, and F_2, could be identified on two and four day exposures of autoradiographs. However, immunoprecipitations using mouse anti-BRSV concentrated hybridoma or ascites fluid never reproduced those results even on 32 day exposures of autoradiographs. Many RIPA SDS-PAGE procedure variables were analyzed including the [^{3}H]glucosamine radioisotope source (manufacturer), radiolabeling pulses, viral passage level, BT cell passage level, acrylamide source, type of gel box, etc., but no improvement in results occurred. Subsequently, autoradiographs of [^{3}H]glucosamine-labeled BRSV-infected BT cell lysates in RIPA SDS-PAGE never adequately provided identification of the G gp.

No Mabs produced were specific to the G gp. Since the G gp can often be identified by SDS-PAGE when [^{35}S]methionine-labeled BRSV-infected BT cell lysates are immunoprecipitated with hyperimmune antisera specific to BRSV, an anti-G Mab might have been identified in [^{35}S]methionine-labeled BRSV-infected BT cell lysates by RIPA SDS-PAGE. To date, there are no reports of a Mab to the G gp of BRSV. Perhaps the G gp is present in reduced quantities in purified virions (Orvell 87). Wunner, *et al* (76) suggested that partial purification was accompanied by the loss of high molecular weight glycoproteins. However, Tsutsumi, *et al* (87) were able to produce Mabs to HRSV G gp using purified HRSV virions, but Klucas, *et al* (88), using a BRSV-infected whole cell

lysate as an immunogen, did not obtain an anti-G Mab. In our study, immunized mice were able to recognize the G gp. However, it is possible that the process of purification and that the conditions used in the RIPA SDS-PAGE assay produced enough alterations in BRSV G gp epitopes to prevent proper identification of Mabs by RIPA SDS-PAGE analysis.

It may be possible to identify the BRSV G Mab using alternative methods. For example, in Western blot analysis viral proteins are separated by SDS-PAGE, incubated with serum or ascites, washed, incubated with an anti-mouse antibody conjugated to an enzyme or radiolabel, washed again, and incubated with a substrate. Another possibility would be to utilize a [^{3}H]threonine-labeling in the RIPA SDS-PAGE analysis. Since the BRSV G gene codes for many threonine residues, a [^{3}H]threonine labeling may better identify a G Mab (Collins 86). Previous work with this radiolabel in this laboratory, however, was disappointing for labeling the G gp (unpublished data). Investigators have radiolabeled other sugars such as mannose but have had difficulty identifying the G gp (Dubovi 82).

Although the identification of antigenic variation in RSV has historically been based on reaction panels of Mabs to several isolates of RSV, other methods exist for defining antigenic variation. A better appreciation of strain variation might be obtained by using Mabs to the biologically important epitopes of the fusion protein (Walsh 86). A similar approach has been used to evaluate strain variation of Newcastle disease virus (Iorio 84). The Rnase A mismatch cleavage technique has been shown to be very sensitive for detecting small changes in RNA sequences that are not detectable by Mabs,

including single-base substitutions (Myers 85); however, it may prove to be too sensitive a technique to distinguish subgroup variation. The cDNA hybridization approach to RSV subgroup determination has the advantage of providing broad-based discrimination of RSV subgroups based on nucleic acid homology without interference from minor antigenic differences detected by Rnase A mismatch assays (Sullender 90). The restriction endonuclease mapping technique is simple, rapid, and more discriminating than analysis using Mabs. One reason to emphasize the Mab approach is that antigenic variation is defined on the immunological level, whereas other approaches define antigenic variation on the nucleic acid level.

CONCLUSIONS AND RECOMMENDATIONS

Conclusions that can be made from this study include:

1. **Virus Purification**: Extracellular virions of BRSV can be purified from BRSV-infected BT cell tissue culture media and from reconstituted SBAH BRSV^R vaccine. When using tissue culture media from BRSV-infected BT cells to purify extracellular virions, media should be harvested at 60 hrs PI to avoid excessive loss of lytic BRSV-infected BT cells from the monolayer into the tissue culture media. Extracellular virions can be recovered from BRSV-infected BT cell tissue culture media and from reconstituted SBAH BRSV^R vaccine in amounts sufficient to immunize mice. To produce panels of Mabs to isolates other than the SBAH BRSV^R vaccine strain, however, extracellular virions must be purified from tissue culture media harvested from BRSV-infected BT cells. The work reported here establishes that method for future use.

2. **Immunization**: Balb/c mice can be immunized to produce antibodies specific to BRSV viral proteins. Mice immunized with lentil lectin coated beads incubated with either tissue culture media harvested from BRSV-infected BT cells or from BRSV-infected BT cell lysates did not produce antibodies specific only to BRSV viral gps. Pelleting extracellular virions by ultracentrifugation of the media or precipitation by PEG

prior to incubation with lectin coated beads may improve results of immunization by the BM method. Using lectin coated agarose beads incubated with BRSV-infected BT cell lysates to select for viral gps was ineffective.

3. **Fusion**: Hybridomas secreting antibodies specific to BRSV can be produced using NS-1 myeloma cells or SP2/0 myeloma cells. Using of SP2/0 myeloma cells for the production of hybridomas is less costly.

4. **Screening**: Indirect immunofluorescence is faster, easier, and more efficient than EIA in screening hybridomas for the production of antibodies specific to BRSV. Hybridomas secreting antibodies specific to BRSV produced two patterns of immunofluorescence dependent upon which viral protein the Mab is directed against.

5. **Characterization of Hybridomas**: Nineteen Mabs reactive to one or more BRSV viral proteins were produced. No Mabs specific to the G or M protein were produced. The [^{3}H]glucosamine-labeled RIPA SDS-PAGE never consistently allowed identification of viral surface membrane glycoproteins in our laboratory.

Recommendations for further studies include:

1. **Immunization**: Extracellular virions from the tissue culture media of BRSV-infected BT cells should be pelleted (by ultracentrifugation) or precipitated (by PEG) prior to incubation with lentil lectin coated beads to decrease the probability of lectin coated beads binding viral gps on intact extracellular virions. Other methods to immunize mice for the production of antibodies specific to BRSV G gp should be examined (see discussion).

2. **Fusion**: All hybridomas that were positive to BRSV-infected BT cells and uninfected control cells by IFA should be subcloned and rescreened. All hybridomas that were positive to BRSV-infected BT cells by EIA and negative by IFA negative hybridomas should be rescreened and characterized by RIPA SDS-PAGE to determine if any clones produce antibodies specific to BRSV viral proteins. The SP2/0 line of myeloma cells should be used for future fusions.

3. **Screening**: To avoid loss of hybridomas producing antibodies specific to BRSV when screening by IFA only, both IFA and EIA should be utilized. A variety of EIA plates should be tested prior to fusion using the tissue culture medium to be utilized during fusion and cultivation of hybridomas.

4. **Characterization of Mabs**: All hybridomas that immunoprecipitated N with either F_1 or P should be subcloned twice by limiting dilution and retested by RIPA SDS-PAGE. If these hybridomas still immunoprecipitate more than one viral specific protein, then radiolabeled RIPA material should be ultracentrifuged (100,00 x g for 45 minutes) or the nonionic detergent Empigen should be added to the buffer prior to SDS-PAGE analysis. Western blot analysis might be used to confirm viral protein specificity of Mabs. Ascites fluid or concentrated tissue culture hybridoma fluid should be purified using standard protocols such as ammonium sulfate precipitation, diethylaminoethyl-cellulose column dialysis, or affinity chromatography (Campbell 84). Virus neutralization and fusion inhibition assays should be repeated using purified ascites or tissue culture hybridoma fluid.

5. **Future studies:** Other methods to determine antigenic variation in BRSV should be examined. These methods might include Rnase A mismatch cleavage analysis, cDNA hybridization assays, or restriction endonuclease analysis.

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