Human humoral immunity to Respiratory Syncytial Virus: Correlates of disease severity and protection

By

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A Thesis
Submitted to the Faculty of Graduate Studies,
University of Manitoba, in Partial Fulfillment
of the Requirements for the Degree of

Master of Science

Department of Medical Microbiology / Infectious Disease

University of Manitoba

Winnipeg, Manitoba

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HUMAN HUMORAL IMMUNITY TO RESPIRATORY SYNCYTIAL VIRUS: CORRELATES OF DISEASE SEVERITY AND PROTECTION

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GARRET DREW WONG

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

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MASTER OF SCIENCE

Acknowledgements

The completion of this degree would not be possible without the aid and support of several people. First, I would like to sincerely thank Dr. Neil Simonsen and Dr. Kevin Coombs for allowing me train in their labs and benefit from their vast experience. Their valuable input has had a great impact on my graduate education.

My thanks goes out to my advisor, Dr. Barbara Law, who has given me the support, patience, and strength to see my degree through to its completion. Her mentor skills have allowed me to develop into a well-rounded student, for which I will always be grateful.

I would also like to thank my friends for all of their support and advice throughout my studies. Further thanks goes out to the staff and students of the department of Medical Microbiology for all of their help.

Finally, I would like to thank my family for all of their love and support. They have instilled in me the desire for success and the strength to achieve it.

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Abbreviations List

1FB	1st F Protein Fragment
2FB	2nd F Protein Fragment
3FB	3rd F Protein Fragment
AmPS	Ammonium persulfate
ATCC	American Type Culture Collection
BPD	Bronchopulmonary dysplasia
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CIP	Calf Intestinal Phosphatase
CO_2	Carbon Dioxide
CPE	Cytopathic Effect
dNTP	Deoxynucleotide Triphosphates
DTT	Dithiolthreitol
EIA	Enzyme immunoassay
ELISA	Enzyme Linked Immunosorbant Assay
FE	Fisher Exact
FCS	Fetal Calf Serum
GMT	Geometric Mean Titer
ICU	Intensive care unit
IPTG	Isopropyl-β-D-thiogalactopyranoside
IVIG	Intraveous immunoglobulin

KCl	Potassium Chloride
KD	Kilodaltons
KH_2PO_4	Potassium Phosphate
LRTI	Lower respiratory tract infection
MEM	Minimal Essential Medium
$MgCl_2$	Magnesium Chloride
MOI	Multiplicity of Infection
Na_2CO_3	Sodium Carbonate
Na_2HPO_4	Sodium Phosphate
$NaHCO_3$	Sodium Bicarbonate
NaOAc	Sodium Acetate
NaOH	Sodium Hydroxide
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RSV	Respiratory Syncytial Virus
RT	Reverse Transcriptase
SDS	Sodium Dodecyl Sulfate
Tm	Melting temperature of nucleic acids
UV	Ultraviolet

Abstract

To better understand humoral immunity against respiratory syncytial virus (RSV) and the role of serum antibodies to the surface fusion (F) glycoprotein in disease severity and protection, a large database consisting of collected sera and patient information was analyzed using plaque reduction neutralization and F antigen ELISA assays. Out of 73 infected and 73 non-infected infants with cardiopulmonary disease (matched for age and underlying disease), neutralizing antibody titers ≥1:100 and ELISA titers ≥1:10,000 were significantly correlated with a decreased incidence of RSV infection. The neutralization and ELISA assays also correlated highly when compared to each other. Acute sera samples from previously healthy infants for whom infecting subgroup was known (82 infected with subgroup A, 127 infected with subgroup B) were also analyzed as above, and an age <13 weeks was found to be significantly associated with severe RSV disease. As well, those infants infected with subgroup B, having an F ELISA titer >1:1000 had a 5 fold less incidence of hypoxia, independent of age. Out of 45 subgroup A infected and 99 subgroup B infected infants and children for which paired acute - convalescent sera were collected, young age was significantly associated with a poor convalescent antibody response. For B infected infants only, neutralizing antibody titers <1:40 against subgroup B were significantly associated with a poor homologous response. Collectively, these results contribute to the understanding of humoral immunity to RSV.

Introduction

Clinical Relevance

Respiratory Syncytial Virus (RSV) is a major cause of respiratory tract infection in infants and children worldwide, infecting 50% of children by one year of age and 100% by two years of age (1). Seasonal outbreaks are the hallmark of RSV, occurring during the winter months in cold climates and the rainy season in tropical climates, when crowding is at a maximum (2). After an incubation period of 4 to 5 days, the virus spreads from the nasopharynx to the lower respiratory tract via aspirated secretions or via contiguous cell to cell spread along the columnar respiratory epithelial mucosa (3). The clinical forms of RSV disease include the common cold, bronchiolitis, bronchopneumonia and pneumonia. Approximately 1 or 2 of every 100 previously healthy infants infected with RSV are hospitalized, but among such infants mortality is less than 1%. Nevertheless, RSV is the only pediatric respiratory pathogen for which seasonal outbreaks parallel infant mortality due to respiratory illness (13). Mortality is significantly increased among infants with underlying pulmonary and cardiac disease and in the immunocompromised (4). The economic impact of RSV is large, estimated at $340 million per year in the United States. (5) and $6500 per hospitalized case in Canada (6). Currently there is no effective treatment for infection, nor effective vaccine.

Molecular Biology of the virus

The enveloped Respiratory Syncytial Virus belongs to the family Paramyxoviridae, in the genus pneumovirus. The nonsegmented negative sense single stranded RNA genome consists of approximately 15,200 nucleotides that encodes two nonstructural proteins (NS_1, NS_2), three

nucleocapsid proteins (N, P and L), two nonglycosylated virion matrix proteins (M, M2) and three transmembrane surface proteins (G, F and SH) (7). The viral replicative cycle occurs in the cytoplasm, where the genomic RNA is transcribed into subgenomic messenger RNAs. Progeny virions bud out of the host cell membrane, acquiring a lipid envelope containing virally encoded outer membrane proteins (8). RSV is unique when compared to other family members due to a helical nucleocapsid diameter of 12 to 15 nm rather than the usual 18 nm and the absence of hemagglutinating and neuraminidase activity, present in most of the genera, including Paramyxovirus and Rubulavirus (9). RSV strains have been assigned to one of two subgroups, A or B, based on reactivity of monoclonal antibodies directed against the G glycoprotein.

Among the 10 RSV proteins, the F glycoprotein (fusion protein) and G glycoprotein (attachment protein) are the best characterized. The F glycoprotein has approximately 79% homology between subgroups A and B at the RNA level, which translates into 89% homology at the protein level. It is related functionally and structurally to the F proteins of the other paramyxoviruses (10,11), and mediates fusion to give the characteristic syncytium formation in tissue culture (12).

F protein is a Type I glycoprotein that contains a cleaved N-terminal signal sequence and a C terminal portion anchored in the membrane. It is synthesized as a precursor F_0, consisting of the F_2 domain (amino acids 1 to 130), the F_1 domain (amino acids 137 to 574), and a cleavage peptide (amino acids 131 to 136) (10). The F_2 and F_1 domains are linked by two disulfide bonds. and chemical and cross-linking studies suggest that the F protein exists as a dimer, effectively making up a tetrameric spike (13). The protein is sparsely glycosylated, partially modified by the addition of N-linked sugars at five or six potential acceptor sites, mostly in the F_2 domain (14).

The G protein has approximately 50% protein homology between the A and B subgroups,

and forms the basis for monoclonal antibody immunological subtyping. It is heavily glycosylated, with carbohydrate sugars responsible for over 40% of its molecular weight, and induces neutralizing antibodies. Relative to the G, the F protein has greater potential as a vaccine candidate, since it induces both neutralizing and fusion inhibiting antibodies, has demonstrated greater protection in animal models and is able to induce cross reactive neutralizing and fusion inhibiting antibodies against both subgroups A and B. (15,16,17,18,19).

Subgroup Specificity

Subgroups A and B usually co-circulate during yearly epidemics, although subgroup A usually predominates (20,21,22,23). A homologous response is defined as the rise in neutralizing antibody to the same subgroup of the originally infecting RSV strain; a heterologous response is the rise in neutralizing antibody against the opposite subgroup. In general, the homologous neutralizing antibody exceeds the heterologous response (22). However, antibody induced by a subgroup A strain neutralizes both A and B strain viruses, whereas subgroup B strains induce a good homologous but poor heterologous neutralizing responses (24).

Functional versus Quantitative Assays

Reactivity of antibodies to RSV can be measured functionally or quantitatively. Functional assays include neutralization and fusion inhibition assays. Quantitative assays measure the amount of antibody and include enzyme immunoassays against various antigens. Siber et al. studied the ability of seven different functional or quantitative assays to identify plasma IgG with high virus-neutralizing and animal protective activity (25). The functional assays included the microneutralization assay and the plaque reduction assay. The quantitative

assays were all ELISA-based and included three direct assays with F, G, or RSV lysate as the antigen and two competitive assays with F antigen and monoclonal antibodies. IgG from plasma units selected by the microneutralization assay had significantly higher protective activity in mice than the other assays. The plaque reduction assay was least able to identify plasma IgG with protective activity in mice, while the quantitative ELISA assay using F protein as the antigen was second only to the microneutralization assay. Interestingly, the assay least able to select for protective activity was also a functional assay. The microneutralization and plaque reduction assays are similar, except the virus and cells are incubated together during the entire experiment in the microneutralization assay, mimicking an in vivo situation.

Role of Humoral Immunity in RSV Infection

In the 1960's, a trial using a candidate formalin-inactivated whole RSV vaccine not only failed to induce protection against RSV, but once infected, vaccinees had more severe disease with higher mortality relative to controls (2,5). Studies with stored sera from vaccinees and animal model reconstructions have suggested the formalin treatment may have inactivated important protective epitopes (26). Sera from vaccinees had high titers of antibodies that bound to the F glycoprotein, but low levels of neutralizing activity. The conclusion from these experiments was that humoral antibody to RSV actually enhanced disease and had a negative impact on the development and testing of new vaccines against RSV. More recently, however, several lines of evidence have suggested a more beneficial role for humoral immune responses in terms of protection against severe RSV infections (27-39).

As yet, no animal model has been described that accurately mimics RSV infection in humans. The cotton rat and the Balb/c mouse have been used extensively since RSV multiplies in

a characteristic, reproducible fashion in both the upper and lower respiratory tract after viral challenge. However, unlike humans, the animals do not develop any symptoms of respiratory illness. Thus the models are less than ideal for exploring the immunity and pathogenesis of human disease. Nevertheless, strong preliminary evidence that humoral antibody plays a protective role in RSV infection was gathered using these models.

In BALB/c mice depleted of B-cells, passively administered RSV-specific immune serum reduces viral lung titer after the first challenge with RSV and is required to prevent severe RSV disease in those rechallenged with the virus (27). Passively administered polyclonal and monoclonal RSV antibodies and pooled human immune globulin for intravenous administration (IVIG) protected cotton rats against challenge with RSV and reduced established RSV infection (28, 29,30,31,32,33,34).

A growing body of evidence from human studies corroborate the animal model data regarding the protective role of humoral immunity. Higher ELISA antibody titers against purified F protein are significantly associated with a reduction in the risk of primary infection and reinfection in children and adults (35). A higher titer of neutralizing antibody in cord sera is associated with a greater time interval between birth and an infant's first RSV infection, and with reduced severity of infection (36,37,38,39). Recurrent infections with RSV occur throughout life, indicating that immunity induced by natural RSV infection is incomplete. However, after two or more infections, neutralization titers increase and stay elevated longer. The net result is reduced severity of subsequent infection, and this phenomenon is independent of age (2,37,41).

In a placebo controlled randomized blinded trial, prophylaxis with a polyclonal human immune serum globulin with a high RSV neutralizing antibody titer (RSV-IVIG) reduced the rates of RSV lower respiratory tract infection (LRTI), hospitalization for RSV-LRTI, admission

to ICU, hypoxemia and viral shedding (37,41). Recently, in a study of similar design, it was shown that the administration of a humanized IgG1 mouse monoclonal antibody that binds to a single site on the F protein reduced hospitalization rates and overall disease severity in premature children with and without bronchopulmonary dysplasia (BPD) (44). Thus, a single monoclonal antibody directed against a single epitope on the F protein is sufficient to protect against severe RSV disease.

Age related human response to RSV

Murphy et al. (45) examined the effects of age and preexisting antibody titer on the humoral response to G and F glycoproteins. Preexisting antibody titer affected the response to the G glycoprotein whereas age was the major factor in the response to the F glycoprotein. Specifically, the F antibody response (as measured by ELISA assay) was significantly lower in children aged 1 to 4 months versus those aged 5 to 8 months. This difference was independent of passively transferred antibody levels. Antibody avidity has also been found to increase with age during the first year of life (46). Pre-term infants born before 28 weeks gestation have significantly reduced cord titers of antibody to the F and G proteins, as well as reduced levels of neutralizing antibody in comparison to term infants. Since most transplacental antibody transfer occurs after 34 weeks gestation these differences were predictable (47).

F Protein epitope research

Research completed during the last decade has focussed on identifying and localizing fusion inhibiting and neutralizing epitopes on the F protein. (15,17,48,50-61). As shown in Figure 1, which summarizes these data, most epitopes are localized on the N terminal third of the

F1 subunit. The task of epitope localization and identification has been approached from three different directions.

The first method consists of producing defined sections of the F protein and measuring the monoclonal and polyclonal binding activity. F protein fragments have been generated as follows: selective chemical or enzymatic cleavage, generation of overlapping nested peptides, synthesis of oligopeptides, and expression of whole or partial recombinant F protein (48,50-52,54-56,58-61). Using such methodology, it has been shown that selected peptides react with RSV-specific polyclonal antisera as well as monoclonal antibodies (48,51,53,55,56,59). The majority of such peptides are located in the F1 subunit of the protein, between amino acids 200 to 300. In addition a few have been localized within the carboxyl terminal region (53).

A second method for identifying epitopes is to assess the binding, neutralizing and animal protective activity of immune sera generated by synthetic peptide vaccines specific to defined regions of the F protein (55,56,59-61). These studies corroborate the localization of specific epitopes as described with the first method.

The third and most precise method of epitope localization uses DNA sequencing of the complementary sequence to the RNA genome to map the location of amino acid changes in monoclonal antibody escape mutants (52,58). Single amino acid substitutions have been shown to confer resistance to neutralizing monoclonal antibody. As seen in Figure 1, most of these changes mapped to a region between amino acids 190 to 300, confirming that many protective epitopes are located in this region.

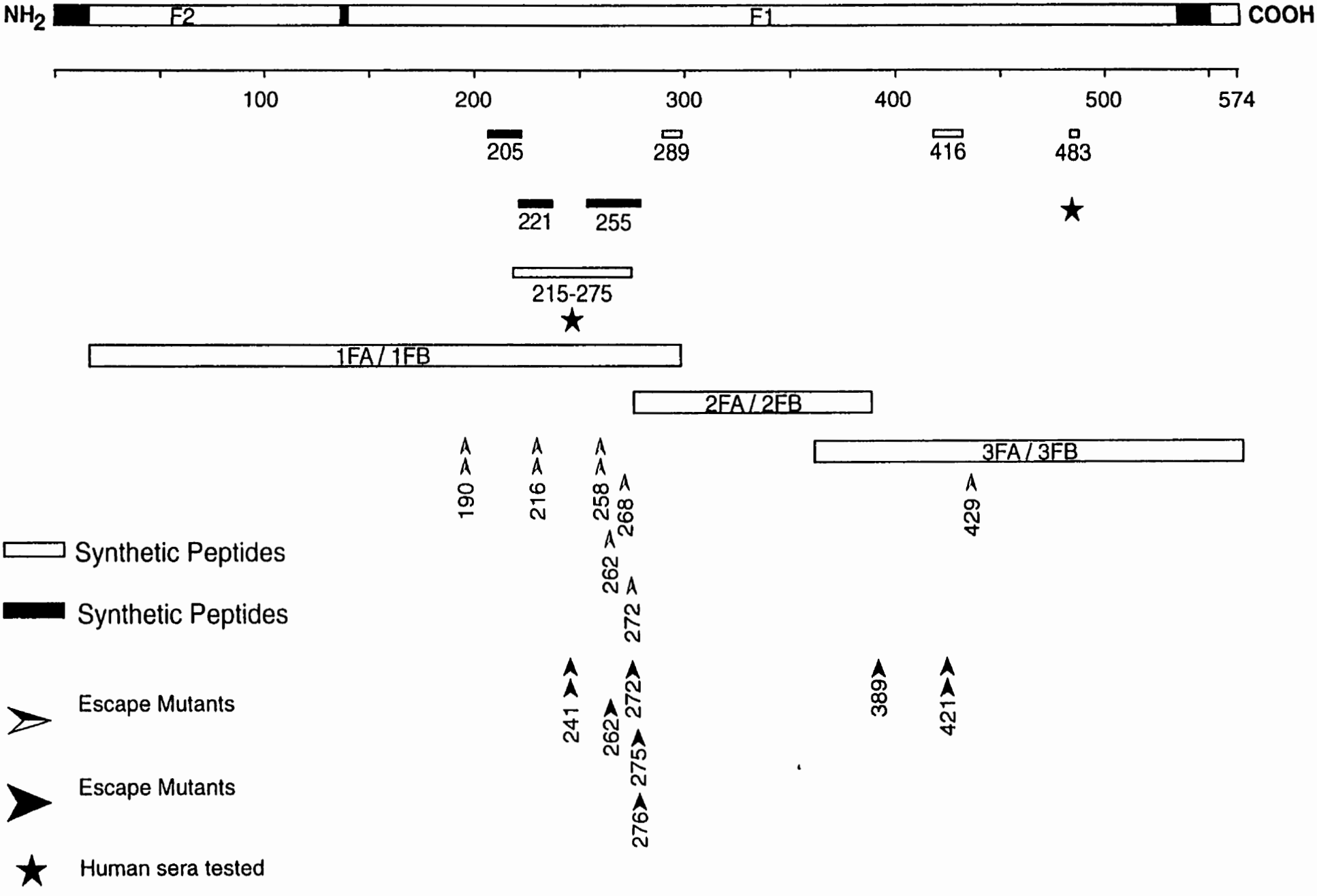

Figure 1. Epitope map of the RSV F glycoprotein. A summary of the recent epitope data for the F protein is illustrated (48-61). Synthetic peptides reacting with antibodies and the sequencing of neutralization escape mutants are the most common methods displayed. Also shown are the proposed overlapping recombinant fragments

With few exceptions, all work to date on F protein epitope localization has been done with animal polyclonal and monoclonal antibodies. Since animals infected with RSV do not develop clinical illness, it is not certain whether protective epitopes in animals have relevance for human infection. Thus, further studies on the human epitope specific antibody responses are needed.

Human response to the F protein

A few groups have reported studies with infant sera and their reactivities to the F protein and smaller molecular weight fragments of the F protein (64,65). Convalescent increases in antibody against the F1 subunit and the whole F protein have been demonstrated (49). In another study, using western blot methodology, the number of fragments recognizing structural protein increased with the number of repeat infections. Although these results suggested an increased range of the antibody response was occurring, the population studied was too small to draw any firm conclusions (64). Levine et al. (65) analyzed acute sera of hospitalized children with western blot, neutralization and fusion inhibition assays. Whole F protein was reactive, and all convalescent sera had evidence of fusion inhibiting activity. When comparing neutralizing activity and reactivity to whole F protein in western blots a small correlation, although not significant, between higher titers (>1:100) and reactivity was found. This was possibly due to the reducing conditions used to electrophorese the proteins, changing the structure, and altering the conformational dependence important for the binding to key epitopes responsible for neutralization and fusion inhibition.

Only two published reports address the reactivity of human convalescent sera to specific

defined regions of the RSV F protein. Lopez et al. (52) synthesized peptides of increasing length and tested them for binding to a panel of neutralizing monoclonal antibodies, rabbit hyperimmune sera, and human convalescent antisera. Most of the 9 human sera reacted with peptides from amino acids 215 to 275 and 235 to 275 but failed to react with a peptide from amino acids 255 to 275. These regions contain most of the neutralization escape mutants mentioned previously, and have been shown to bind several murine monoclonal antibodies with high neutralizing and fusion inhibiting activity (60,61). The smallest fragment (a.a. 255-275) did not react with the sera suggesting that the shortened peptide was unable to express an intact epitope. This study demonstrated that human sera do recognize epitopes in the N-terminal third of the F protein. In contrast to previously determined epitopes, Scopes et al. (53) showed that convalescent human sera recognizes peptides in the C-terminal portion of the F1 subunit. Specifically, a nested set of overlapping decameric synthetic peptides spanning the entire mature protein were produced on polyethylene pins and examined for reactivity with convalescent sera from 4 children of unknown age recovering from RSV infection of unknown severity (53). The sera bound to the amino acid region 483-488, and amino acid substitutions at sites 484 (loss of proline) and 487 (loss of glutamine) led to almost complete loss of binding. The epitope characterized is most likely a linear epitope. The site is remote from the other characterized epitope sites, although they were derived mostly from animal data. Both of the above studies are flawed by the small sample size and lack of relevant clinical information.

Treatment and Prevention

The only current treatment for RSV is supportive, and includes maintenance of hydration, removal of excess secretions, oxygen supplementation, and occasionally mechanical ventilation.

Immunotherapy with RSV-IVIG reduces the levels of pulmonary virus in animals, but to date has been ineffective in humans (30,34). Given the successful demonstration that prophylaxis with RSV-IVIG can reduce the severity of infection in high-risk children the product marketed as Respigam® was recently licensed. However, at a cost of $5000/child/season its role will be limited to a select few.

Vaccine-induced protection is key to the control of infection, and would be far more cost effective. However, despite years of intense research since RSV was first isolated in 1956, an effective vaccine is still not available. Because the majority of RSV infection occurs between the ages of two and seven months, an effective immunotherapy has to be administered and stimulate effective protection before the second month of life (71,72). The formalin-inactivated vaccine experience has made the regulatory authorities extremely cautious in allowing infants and children to be the subjects of test candidate subunit vaccines containing one or more viral proteins. Thus, a better understanding of human immunity to RSV, and more specifically of the role of epitope specific antibody in human infection, is necessary to help overcome these hurdles.

Derivation of sera available for project

An entire databank consisting of collected sera, patient information, and neutralization titers was available, and served as the foundation for this project. Sera was collected from two groups of infants and children enrolled in two large multicenter Canadian studies carried out from January 1, 1993 to June 30, 1995 (75,76). The study groups are described below.

(1) Asymptomatic children less than 36 months of age with underlying lung and cardiac disease, were followed prospectively as outpatients for the development of symptomatic RSV respiratory infection. Children were enrolled prior to the RSV season at each of eight

pediatric tertiary care centers across Canada. Follow-up for the development of any symptomatic respiratory infection was conducted by trained nurses. The endpoint of the study was the end of the RSV season, or symptomatic RSV infection proven by a positive RSV culture or antigen test, collected from a nasopharyngeal aspirate. Sera were collected in the fall prior to any RSV activity and at the end of the annual outbreak for those who never developed RSV illness, or 4 to 6 weeks after proven infection for those who did. The study showed that a pre-season neutralizing antibody titer of >1:100 was associated with a lower risk of subsequent RSV infection in this high-risk group.

(2) Hospitalized RSV-infected infants and children with acute lower respiratory tract infection were enrolled on the first or second hospital day. Data on medical history, presenting signs and symptoms, home environment, clinical disease severity and hospital course were recorded. Acute sera were collected during the first few days in hospital and when possible, convalescent samples were drawn 4 to 6 weeks after the acute illness. The purpose of the study was to identify host viral and antibody related correlates and severity of RSV infection. Also, the purpose was to collect paired sera to assess the age and subgroup specific antibody responses to RSV. A total of 392 paired and 259 single acute sera were collected and tested for neutralizing activity using a 60% plaque reduction neutralization assay and representative strains of both subgroup A (A2) and subgroup B (18537) RSV. A single technologist performed all assays. There were a total of 121 subgroup A and 330 subgroup B infections identified for which single acute sera were collected from 48 and 146 respectively, and paired convalescent sera from 73 and 184 respectively. It was shown that early hypoxemia (defined as an oxygen saturation of <90% while breathing room air, within 48 hours of hospital admission) was significantly correlated with severe disease as measured by admission to intensive care, need for ventilation and

prolonged duration of hospital stay.

Research Goals

The goal of this research project was to contribute to the understanding of the human humoral immune response to RSV by characterizing epitope specific human antibody responses to infection.

The specific objectives included:

(A) To measure the ELISA antibody to the F protein in each group of sera in order to:

(1) Describe the acute and convalescent change in titer and examine the effect of age, prior antibody, infecting subtype, and illness severity on the F response by otherwise healthy children.

(2) Examine the relationship between levels of F protein antibody in acute sera and disease severity for otherwise healthy children.

(3) Examine the relationship between levels of F protein antibody in pre-season sera with frequency of subsequent RSV infection in a group of high-risk children.

(4) Correlate antibody titers determined by the F protein EIA and the plaque reduction neutralization assay.

(B) To clone and express three separate overlapping recombinant fragments of the F protein, encompassing all of the known B-cell epitopes as further detailed in the Materials and Methods section.

(C) To use the previously characterized sera and the expressed F protein fragments in an EIA assay to test the following hypotheses:

(1) For children aged less than 36 months with underlying cardiopulmonary disease, the

titer of antibodies against a specific region of the F protein correlated with the risk of subsequent RSV infection.

(2) For children hospitalized with acute RSV LRTI, the titer of antibodies against a specific region of the F protein is correlated with disease severity as indicated by early hypoxia.

(3) Among a hospitalized population of previously healthy children with acute LRTI due to a known RSV subgroup, the convalescent antibody response to F protein fragments is correlated with age, subgroup, and pre-existing antibody.

It was hoped that data obtained from these studies would provide information that would enhance the understanding of the human humoral immune response to RSV infection.

Materials and Methods

Growth and maintenance of HEP-2 cells

Hep-2 human cells (ATCC #CCL-23) were grown at 37 °C, 5% CO_2 in 5% MEM (minimal essential medium with Earle's salts containing 5% fetal calf serum (FCS) and L-Glutamine at a final concentration of 2mM) in T-150 cell culture flasks (Corning) until 90% confluency was achieved. Cells were then harvested by removing the entire medium from flask, rinsing the flask with 5-10 mls of MEM without FCS, and rinsing the flask with 5 mls of Trypsin/EDTA (0.002% EDTA, 0.21% sodium bicarbonate, pH 7.3). After discarding, 5 mls of fresh Trypsin/EDTA was placed on the monolayer and the flask was incubated at 37° C for 3 minutes. The Hep-2 cells were dislodged by lightly bumping the flask, and then 10 mls of 5% MEM was immediately pipetted into the flask to deactivate the trypsin. The entire contents were transferred into a 15 ml centrifuge tube, and centrifuged for 10 minutes at 4° C, 300 x g. The cell pellet was then gently resuspended in 5 mls of 5% MEM with glutamine and split into new flasks (generally, 3 x 10^6 total cells were seeded into each flask), each flask supplemented with 30 mls of fresh completed 5% MEM with glutamine.

Growth, amplification and titer of RSV A2 and 18537 strains

T-150 tissue culture flasks were seeded with 3.75 x 10^5 cells one day before infection. On day of infection, virus stocks (A2 and 18537 strains of RSV, obtained from ATCC #VR-1302 and #VR-1401, respectively) were taken from storage at -80° C, placed on ice, thawed quickly in a 37° C waterbath, then placed on ice again. A dilution was made (according to previous titer) in

2% MEM for a Multiplicity of Infection (MOI) of 1 (i.e. 1 virus particle per cell). Media was aspirated from the HEp-2 cells, and 4 ml of viral inoculum was loaded onto each monolayer. The flasks were then incubated at 37° C, 5% CO_2 for 2 hours rocking the flasks every 30 minutes to moisten the monolayer, after which 25 ml of 2% MEM was added and incubated overnight at 37°C, 5% CO_2. On day 2, the media was aspirated, 14 ml of 0.5% MEM was added to each and the flasks were further incubated for 2 days in the same conditions as noted above. When CPE (cytopathic effect) was observed (12), generally on day 4, each flask was scraped with a rubber spatula, and all media and cells were poured into centrifuge tubes and spun at 300 g, 10 minutes at 4°C. The supernatant was decanted into a separate container, and the cell pellet was subjected to 3 freeze thaw cycles (i.e. dry ice, 37°C waterbath, vortex, repeated 3 times). The pellet was centrifuged at 600g for 20 minutes at 4°C. The supernatant was then combined with the previous supernatant, aliquotted into vials, flash frozen (dry ice / ethanol bath) and stored at -80°C.

Plaque reduction neutralization assay

The protocol for the neutralization bioassay was obtained from Lederle-Praxis Biologicals (Standard serologic assay #CA-5). The assay was utilized by a single research technician, who tested the entire banks of sera (as outlined in the introduction).

F protein enzyme-linked immunosorbant assay

In order to quantitate the amount of antibodies to the F glycoprotein, an ELISA assay was performed where human sera at varying dilutions were added to microtiter plates with F protein pre-absorbed to them. The immuno-affinity purified F protein (isolated from the A2 strain of RSV) was generously donated by Wyeth-Lederle Vaccines. The amount of antibody bound is

proportional to the amount of color development (protocol received from Wyeth-Lederle Vaccines and Pediatrics). This previously published protocol was chosen to achieve consistency for further comparison to other published studies. Briefly, 20 ng of F protein (diluted in a solution of sterile 0.05M $Na_2CO_3/NaHCO_3$, pH 9.6) was added to each well in a polystyrene 96 well microtiter plate (polysorp plates from Nunc Inc.) and incubated for 2 hours at 37° C. Sera were run in duplicate, serially diluted 3 fold in PBS-Tween (0.009M $Na_2HPO_4 \cdot 7H_2O/KH_2PO_4$, 0.139M NaCl/KCl, 0.3% Tween20, pH 7.2) containing 0.01M EDTA, starting from 1:150 in row A to 1:328050 in row H (50 µl of diluted sera loaded per well). Respigam (human hyperimmune immunoglobulin, obtained from MedImmune Inc.) was used as a standard control on every plate, diluted from 1:5000 to 1:36450000. Sera was added and incubated for 1 hour at room temperature after the plates had been washed on an automated plate washer (Wellwash 12) with PBS-Tween for 4 cycles and blotted on paper towels. After washing again for 4 cycles, the secondary antibody was added (goat anti-human IgG alkaline phosphatase conjugate, Jackson Inc.), diluted 1:2500 in PBS-Tween to a final concentration of 2.5 µg/ml (100 µl per well) and incubated for 60 minutes at room temperature. After washing the plates again 4 times, 100 µl of the substrate solution was added per well (1 mg/ml p-nitrophenol phosphate in 1 M Diethanolamine/0.01% $MgCl_2$) and incubated for 1 hour at room temperature. The reaction was stopped by adding 50 µl of 3N NaOH to all wells and the plates were read using a Titertek ELISA reader at a 405-410 nm test filter, using a 630-690 nm reference filter. To analyse the data, a log/log linear regression model was used. The average absorbance of the duplicate blank wells was subtracted from the absorbance of all other wells, and the data of each plate was analyzed against the Respigam standard run on each plate. The absorbance values between 0.02 and 2.0 (log) of each sera was plotted versus the reciprocal of the dilution (log), the best fit linear

line was drawn, from which the dilution of serum yielding an absorbance reading of 0.2 was determined. All graphs and calculations were performed on Microsoft Excel 5.0. All data was normalized to a value of 66,100, determined by measuring the titer of Respigam on 8 separate plates, repeated on 5 separate occasions, and averaging all values. All titers below the cutoff of 1:150 were assigned a value of 1:75.

RNA template preparation and extraction

Respiratory syncytial virus was absorbed onto subconfluent monolayers of HEp-2 cells (80%) in 35 mm dishes at 37° C, 5% CO_2 for 2 hours, after which 3.5 ml 2% MEM was added and further incubated overnight in the same conditions. The next day, the media was replaced with 1.5 ml 0.5% MEM, incubated until CPE was evident (day 4 or 5), and the cells and virus were scraped into the medium with a rubber policeman and transferred into 1.5 ml polypropylene tubes (on ice). Tubes were centrifuged at top speed in a microcentrifuge for 2 minutes at 4° C. and the supernatant was aspirated. The virus/cell pellet was then subjected to a Hot Phenol extraction (73). Briefly, 0.5 ml 4M Guanidinium Isothiocyanate (containing 0.05 M TRIS-HCl, pH 7.6, 0.001M EDTA, pH 8.0) was added at 65° C and vortexed well. 0.5 ml of saturated Phenol (pre-warmed to 65° C) was added and vortexed vigorously (phenol saturated with 0.1 M sodium acetate, pH 5.2). 0.2 ml of 0.1M NaOAc pH 5.2, 10mM TRIS, 1mM EDTA was added at 65° C, material was vortexed and split into two tubes, and 0.3 ml of choroform was added, vortexing intermittently for 10-15 minutes, keeping the temperature at 65° C. The sample was then cooled to 4° C, and phases were separated by spinning for 10 minutes, full speed in a refrigerated eppendorf centrifuge. The upper aqueous phase was removed to another tube, and 0.6 ml of a saturated phenol:chloroform mixture (1:1) was added. The extraction was repeated

two more times, and the sample was then extracted twice at room temperature with an equal volume of chloroform. RNA was precipitated by adding two volumes of ethanol, storing the sample overnight at -20° C, and spinning full speed in refrigerated centrifuge for 30 minutes. Ethanol was aspirated and sample was dried in a vacuum dessicator for 30 minutes. The RNA pellet was then dissolved in 30 µl DEPC H_2O and 20 units of RNase inhibitor.

Design of reverse transcriptase and PCR primers

The template for amplification was the single stranded cDNA generated by reverse transcription of the mRNA from each of strains A2 and 18537 using an oligo dT primer. Primer sets were designed to yield the entire F protein in three overlapping segments of the A2 and 18537 strains. The fragments were determined by choosing the best regions that represented all of the known B-cell epitopes as detailed in the introduction (see Figure 1). Each oligonucleotide (forward and reverse primers) had three unique restriction endonuclease sites engineered into them, as well as a 20 base region complementary or corresponding to the open reading frame (ORF) of the F protein (Figure 2a and 2b).

Reverse transcription of RNA into cDNA template

Reverse transcriptase was performed using RNA obtained from the A2 and 18537 strains of RSV with an oligo d(T) primer to start the reaction. The cDNA was then amplified using polymerase chain reaction (PCR) utilizing the 6 pairs of oligonucleotides outlined above.

Fragment	Primer set	Amino Acids	Predicted Mlc Wt. (KD)
1FA	**1fproA5'** – 5' TAGGATCCAAGCTT*CATATG*GAGTTGCTAATCCTC 3' (corresponding to positions 1 to 18 of the open reading frame (ORF)) (restriction enzymes BAMHI in bold, HINDIII underlined, NDEI in italics). **1fproA3'**-5'ATGAATTCAGATCT*CATATG*TCACTAAGACTTCCTCTTTTATT 3' (complementary to positions 873 to 892 of the ORF) (restriction enzymes ECORI in bold, BGLII underlined, NDE I in italics).	1-297	33
2FA	**2fproA5'** – 5' TAGGATCCAAGCTT*CATATG*TCCAACAATGTTCAAAT 3' (corresponding to positions 820 to 839 of the ORF) (restriction enzymes BAMHI in bold, HINDIII underlined, NDEI in italics). **2fproA3'** – 5' ATGAATTCAGATCT*CATATG*TAATATGTCAACATTGCAGAG 3' (complementary to positions 1141 to 1158 of the ORF) (restriction enzymes ECORI in bold, BGLII underlined, NDE I in italics).	274-386	13
3FA	**3fproA5'** – 5' TAGGATCCAAGCTT*CATATG*AAAGTTCAATCAAATCGAG 3' (corresponding to positions 1075 to 1093 of the ORF) (restriction enzymes BAMHI in bold, HINDIII underlined, NDEI in italics). **3fproA3'** – 5' ATGAATTCAGATCT*CATATG*TCAGTTACTAAATGCAATAT 3' (complementary to positions 1706 to 1725 of the ORF) (restriction enzymes ECORI in bold, BGLII underlined, NDE I in italics).	359-574	24

Figure 2a. Oligonucleotides designed for the amplification of three separate F protein fragments from the open reading frame of the A2 strain of RSV.

Fragment	Primer set	Amino Acids	Predicted Mlc Wt. (KD)
1FB	**1fproB5'** – 5' TAGGATCCAAGCTT*CATATG*GAGTTGCTGATCCAC 3' (corresponding to positions 1 to 18 of the open reading frame (ORF)) (restriction enzymes BAMHI in bold, HINDIII underlined, NDEI in italics). **1fproB3'** – 5' ATCCCGGGCTGCAG*CATATG*TCACAAGGACTTCTTCCTTTATT 3' (complementary to positions 873 to 892 of the ORF) (restriction enzymes SMAI in bold, PSTI underlined, NDEI in italics).	1-297	33
2FB	**2fproB5'** – 5' TAGGATCCAAGCTT*CATATG*TCAAGCAATGTTCAGAT 3' (corresponding to positions 820 to 839 of the ORF) (restriction enzymes BAMHI in bold, HINDIII underlined, NDEI in italics) **2fproB3'** – 5' ATGAATTCAGATCT*CATATG*TAATATGTCAGTGTTACAAAG 3' (complementary to positions 1141 to 1158 of the ORF) (restriction enzymes ECORI in bold, BGLII underlined, NDEI in italics).	274-386	13
3FB	**3fproB5'** – 5' TAGGATCCAAGCTT*CATATG*AAAGTACAGTCCAATCGAG 3' (corresponding to positions 1075 to 1093 of the ORF) (restriction enzymes BAMHI in bold, HINDIII underlined, NDEI in italics). **3fproB3'** – 5' ATGAATTCAGATCT*CATATG*TCATTTGCTGAATGCAATAT 3' (complementary to positions 1706 to 1725 of the ORF) (restriction enzymes ECORI in bold, BGLII underlined, NDEI in italics).	359-574	24

Figure 2b. Oligonucleotides designed for the amplification of three separate F protein fragments from the open reading frame of the 18537 strain of RSV.

Specifically, reverse transcription was carried out using a reaction volume of 100 µl, containing 5 µl RNA, 40 units of RNase inhibitor, dNTP mixture (final concentration 10mM of each nucleotide), supplied Supercript RT buffer, 0.1M DTT, 1 µM oligo d(T) primer, 100 µg/ml BSA, 10 units of reverse transcriptase enzyme (Superscript RT, Gibco, Inc.), and DEPC H_2O. The mixture was incubated for 2 hours at 45° C, and stored at -20° C as a cDNA template for PCR.

General DNA amplification protocol

PCR was carried out using an adapted protocol (73). Forward primer (final concentration 1µM), reverse primer (final concentration 1µM), TAQ buffer (containing 100 mM TRIS-HCl, pH 8.3, 500 mM KCl, 1% gelatin), dNTP mix, 1.5 mM $MgCl_2$, 15 µl cDNA template, and 2.5 units TAQ polymerase (Perkin Elmer) were added to a 0.6 ml eppendorf tube, diluted in sterile ddH_2O to a final volume of 100 µl. Because the oligonucleotides used were designed to insert several extraneous restriction endonuclease sites, the section of primer actually corresponding or complementary to the template was approximately half the size of the oligonucletide. Therefore, a general strategy was devised of two separate annealing temperatures, with 10 cycles at the lower annealing temperature, and 25 cycles at the higher annealing temperature to increase specificity of PCR product. The PCR mixture was subjected to 93° C for 2 minutes, then 10 cycles of 94° C at 2 minutes, 1 minute at 45° C, and 1 minute at 72° C, and then 25 cycles of 94° C for 2 minutes, 60° C for 1 minute, and 72° C for 1 minute, finishing with a final 72° C for 7 minutes, followed by a reduction in temperature to 4° C indefinitely. In this example, annealing temperatures of 45° C and 60° C were derived by calculating the portion of the oligonucleotides

annealing to the cDNA at that instance, calculating the Tm's, and subtracting 5 ° C. Tm's were calculated using the following formula:

$$Tm = (69.3 + 0.41\ (\%G+C\ )) - 650 / \#\ bases$$

Agarose gel electrophoresis and DNA extraction

To screen amplification PCR products, 5% of each PCR reaction was electrophoresed through a 1% (w/v) agarose gel at 10-15 V/cm until the bromophenol blue tracking dye (in the sample buffer, containing .04% bromophenol blue, .04% xylene cyanol FF, 2.5% ficoll and 0.5 μg/ml ethidium bromide) had migrated to the bottom of the gel slab. Also included was a 1 kilobase DNA standard (Gibco Life technologies). Gels were photographed in the dark under UV light using Polaroid 667 film.

In order to purify digested DNA (in preparation for cloning), the DNA/enzyme mixture was electrophoresed through a 1% (w/v) agarose gel, cut out under minimal UV light with a sterile disposable razor blade, sliced into small pieces and stored in 1.5 ml eppendorf tubes in preparation for DNA extraction. To extract the DNA, a DNA purification and extraction kit was used (Boehringer Manheim, Inc.), and a small portion (2%) of the recovered sample was again electrophoresed through a 1% agarose gel to visualize recovery and estimate the concentration of DNA.

General digestion of DNA using restriction endonucleases

All templates were dissolved and stored in either ddH_2O or $T_{10}E_1$ (10 mM TRIS, 1 mM EDTA). For plasmids, 6 μg of DNA was digested in a solution containing the recommended stock enzyme buffer diluted in ddH_2O (typically from Boehringer Manheim) and 50 units of

enzyme, incubated at 37° C for two hours. For PCR products, the DNA product was removed directly from the reaction tube by inserting a 200 µl pipette tip through the layer of mineral oil and withdrawing 90% of the layer underneath. The extracted PCR product was then treated with proteinase K (73). Briefly, the PCR product was extracted once with phenol:chloroform and once with an equal volume of chloroform. This was then mixed in a buffer containing a final concentration of 5 mM EDTA, 10 mM TRIS (pH 8.0), 0.5% SDS and 50 µg/ml proteinase K. The mixture was incubated at 37° C for 30 minutes, heated to 68° C for 10 minutes, extracted once with an equal volume of phenol:chloroform and once with an equal volume of chloroform. One tenth volume of 3M sodium acetate and two volumes of 100% ethanol were added and the DNA was precipitated either for 10 minutes on dry ice, 45 minutes at -80° C or overnight at -20° C. The DNA was spun full speed in a microcentrifuge at 4° C for 20 minutes, washed in 70% ethanol, dried in a speedvac for 20 minutes and dissolved in 80 µl ddH_2O in preparation for restriction enzyme digestion. The DNA would then be digested two hours at 37° C in the recommended enzyme buffer, diluted in sterile ddH_2O, with 50 units of enzyme.

In some cases, when only one enzyme was used for cloning, the target was dephosphorylated with CIP (calf intestinal phosphatase) to remove phosphates, thereby inhibiting the linearized plasmid from re-ligating (73). Briefly, the 6 µg of digested plasmid would be gel purified and resuspended in a 20 µl volume. 18 µl (with 2 µl saved as an un-CIP'd control) was added to the reaction (100µl total containing CIP buffer, 1 unit CIP), incubated at 37° C for 1 hour, inactivated in the presence of 5 mM EDTA, pH 8.0 for 1 hour at 65° C, then extracted once with an equal volume of phenol:chlroform and once with an equal volume of chloroform. The mixture was precipitated with one-tenth volume of 3M NaOAc, pH 7.0 (to prevent the EDTA

from precipitating), two volumes of 100% ethanol, and frozen overnight at -20° C.

Ligation of target DNA with plasmid DNA

Generally, all ligations were carried out with directional inserts (using two different enzymes), eliminating the need to reduce cloning background via CIP treatment. All experiments included a control consisting of plasmid without target DNA to determine cloning background and efficiency. Through trial and error, it was found maximum efficiency (i.e. number of recombinant colonies) occurred when a plasmid to insert molecular weight ratio of 1:3 was used. In a 0.6 ml eppendorf tube, 100 ng of plasmid DNA, the proportional amount of target DNA, Gibco BRL ligation buffer (containing 50mM TRIS-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% (w/v) PEG-8000), sterile ddH_2O and 0.5 units of T4 DNA ligase in a total reaction volume of 20 µl was found to give the best ligation results. The reaction was carried out on ice at all times, optimizing efficiency by heating the DNA / water mixture to 45° C for 5 minutes, snap-cooling on ice and adding ligase buffer and T4 DNA ligase immediately afterwards. The mixture was then incubated overnight in a 15° C waterbath.

Preparation of *E. Coli* for electroporation

Cell preparation was performed as per the manufacturer's instructions (Biorad, Inc.). Briefly, XL1Blue *E. coli* (chosen for stability) were grown in a fresh overnight 10 ml culture in LB media and then inoculated 1/100 volume (10 ml) into 1 liter of LB media. Cells were grown at 37° C with shaking to a density of approximately 0.6 OD (at 600 nm). The cells were then chilled on ice for 15 to 30 minutes, centrifuged in a chilled rotor for 15 minutes at 4000 x g, and resuspended in 500 ml ice cold sterile water. The cells then went through two more washing

cycles, reducing the amount suspended each time to 20 ml and 2 ml respectively (cell concentration should be about 1 –3 x 10^{10} cells/ml). Suspended cells were frozen on dry ice in 40 µl aliquots and stored at -80° C.

Electro-transformation and plating

Electroporation was performed as per the manufacturer's instructions (Biorad, Inc.). Briefly, cells were taken out of storage, placed immediately on ice and allowed to thaw gently. Included with every electroporation were a positive control (native un-cut target plasmid DNA at 1 ng/µl) and a negative control (sterile ddH_2O). 1 µl of each mixture was pipetted into each 40 µl cell suspension, mixed gently with a pipette tip and transferred to its respective chilled 0.1 cm electroporation cuvette. A Bio-Rad Gene Pulsar apparatus was set at 25 µF, pulse controller set at 200 Ω, with electroporation occurring at 1.80 kV. Immediately after electroporation, 1 ml of ice-cold SOC (2% bactotryptone, 0.5% bactoyeast extract, 0.05% NaCl, with KCl, $MgCl_2$ and glucose added to a final concentration of 2mM, 10mM and 20mM respectively) was added to the cuvette, mixed gently, transferred to a 1.5 ml polypropylene tube, incubated for 1 hour at 37° C with shaking, and 5% to 10% was plated onto LB-amp plates (LB-agar plates supplemented with 100 µg/ml ampicillin) and incubated overnight at 37° C.

Screening of potential recombinant clones

Once it was established the ligation and transformation were a possible success, it was necessary to screen for recombinant clones. This procedure was not implemented unless the cloning efficiency was thought to be greater than 85% (i.e. the 'background' on the negative ligation control was no more than 15% of the total colonies counted on the test plate). A number

of colonies (usually between 10 and 20) were inoculated into 5 ml LB media overnight cultures using asceptic technique in prepration for a miniprep alkali lysis protocol, adapted from previously published protocols (73). The pellet from 1.5 ml of bacterial culture was resuspended in 200 µl 50mM Tris / 10 mM EDTA, pH 8.0, and 200 µl of a fresh 0.2M NaOH / 1% SDS solution was added and the tube inverted gently several times. 200 µl chilled potassium acetate (2.8M, pH 5.2) was added, the tube was inverted several times, placed on ice for 10 minutes and spun full speed in a microfuge. 1 ml 100% ethanol was added to the supernatant and the DNA was precipitated by freezing for 10 minutes on dry ice, 45 minutes at -80° C or overnight at –20 ° C. The DNA pellet was recovered by centrifuging at full speed in a microfuge at 4 ° C. washing in 70% ethanol and resuspending the dried pellet in 50 µl sterile ddH_2O. 15 µl of each clone was subjected to restriction enzyme digestion (with the same endonucleases used to clone in the original target DNA) in the presence of RNase A (final concentration 0.17 mg/ml), 5 units of each enzyme, compatible enzyme buffer and sterile ddH_2O. After incubation at 37° C for 2 hours, the entire 20 µl digest was electrophoretically analyzed on a 1% agarose gel with an uncut plasmid control in the presence of ethidium bromide and visualized under UV light.

Confirmation of insert via sequencing

To confirm the sequence of the target insert, cycle sequencing was performed using a protocol adapted from a cycle sequencing kit (Gibco Life Technologies), implementing the Sanger method (77). Briefly, sequencing plates were cleaned thoroughly with soap and water, scraped with a razor blade, rinsed in warm water, rinsed with 95 % ethanol and wiped with Kimwipes. The inside plate was coated with dichlorodimethylsilane, wedge spacers were inserted between the plates and the edges were clamped around the periphery. A 5% UREA gel

(containing 5% acrylamide:bis, 42% Urea, TBE, temed and AmPS) was poured and allowed to polymerize overnight with an inverted shark's tooth comb in place. 2 overlapping forward primers and 2 overlapping reverse primers (1 pmol of each) were labelled at 37° C for 10 minutes in the presence of 1 unit T4 Polynucleotide Kinase, kinase buffer, and 2 pmol of [γ-^{32}P]ATP, stopping the reaction at 55° C for 5 minutes. Each labelled primer was then separated into 4 tubes, and termination mixes were added (A: 2 mM ddATP and 100 μM each dATP, dCTP, 7-deaza-dGTP, and dTTP; C: : 1 mM ddCTP and 100 μM each dATP, dCTP, 7-deaza-dGTP, and dTTP; G: 0.2 mM ddGTP and 100 μM each dATP, dCTP, 7-deaza-dGTP, and dTTP; T: : 2 mM ddTTP and 100 μM each dATP, dCTP, 7-deaza-dGTP, and dTTP), along with TAQ buffer, 1.5 mM $MgCl_2$, 2.5 units of TAQ polymerase, 15 to 50 fmol of template DNA and a top layer of mineral oil. The tubes were heat cycled at 95° C for 3 minutes, then 20 cycles at 95° C for 30 seconds, 48° C for 30 seconds, 70° C for 30 seconds, then 10 cycles of 95° C for 30 seconds, and 70°C for 60 seconds. 5 μl of stop solution (95% (v/v) formamide, 10 mM EDTA (pH 8.0), 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol) was added to each reaction tube and 15% of each (2.5 μl) was loaded onto the sequencing gel and run at 70 DC watts (approximately 1100 volts) until the bromophenol blue dye had reached the bottom of the plate. The gel was then carefully removed and fixed in a solution containing 5% (v/v) acetic acid and 5% (v/v) methanol, dried on filter paper in a gel dryer and exposed on autoradiograph film for at least 24 hours. The sequence was read (forwards and reverse) manually and compared to the known published sequence (10).

Amplification and screening of mini-preps

To test if the recombinant clones could express recombinant protein, an induction experiment was performed. 5 ml overnight LB-ampicillin cultures were inoculated and used the next day to inoculate 4 ml LB-amp cultures (one tenth volume). After shaking at 37° C for one hour, half the culture (2 ml) was induced by adding IPTG to a final concentration of 0.1 mM and incubating another 2 hours. 15 µl of each culture pellet (induced and un-induced) was denatured in buffer and heated to 95 °C for 5 minutes, then electrophoresed through a Bio-Rad mini-gel apparatus using a discontinuous Laemmli system (78) of 10% polyacrylamide (150 volts, 45 mins), and visualized with Coomassie Blue stain.

SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

To set up the Bio-Rad mini-gel system, all glass plates were soaked in KOH for a minimum of 30 minutes, washed thoroughly in warm water, rinsed in 95% ethanol and allowed to air-dry. Plates were set up according to the manufacturer's instructions (Biorad, Inc.) and the resolving gel (bottom layer) was poured after adding 0.07% TEMED to the gel solution containing buffer I (0.375 mM TRIS-HCl, pH 8.8), 10% acrylamide:Bis (45:1:2), ddH_2O, 0.1% SDS and 0.07% ammonium persulfate. The resolving gel was allowed to polymerize under a solution containing bufferI, 0.1% SDS and ddH_2O. A stacking layer consisting of buffer II (0.125mM TRIS-HCl, pH 6.8), 5% acryl:bis (45:1:2), ddH_2O, 0.1% SDS, 0.07% AmPS and 0.07% TEMED was poured on top of the resolving layer (after rinsing with ddH_2O), and allowed to polymerize with a 10 well comb placed between the glass plates.

All samples were mixed in sample buffer (containing 50 mM TRIS-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), heated to 95° C for 5 minutes, snap-

cooled on ice and loaded into the wells of the polyacrylamide gel. Included also was a Bio-rad low molecular weight protein standard (from 14.4 KD to 97.4 KD). The entire apparatus was subjected to 150 volts for 45 minutes (until the blue tracking dye had reached the bottom), the plates were carefully taken apart and the resolving gel was stained in Coomassie Blue (containing 0.25% coomassie blue, 90%methanol, 10% glacial acetic acid) for 1 hour with shaking, and destained in solution I (containing 50% methanol, 10% acetic acid) for 1 hour with shaking, and solution II (containing 5%methanol, 7% acetic acid) for 1 hour with shaking.

To preserve the gel, a cellophane 'sandwich' was soaked in ddH_2O, stretched in 2 plexiglass frames with clamps around its periphery and allowed to dry overnight.

Determination of protein solubility

To determine whether expressed protein was soluble, the bacterial pellet from a 1 ml culture was resuspended in one-tenth volume $T_{10}E_1$ along with 100 μg ml fresh lysozyme and triton X-100 (final concentration 0.1%) and allowed to stand at room temperature for 15 minutes. The mixture was sonicated twice on ice (microtip at 30%, 10 seconds) and centrifuged at 12,000 g for 2 minutes. The supernatant (soluble fraction) was diluted 1:1 with sample buffer, heated for 5 minutes at 95° C and electrophoresed through an SDS-PAGE gel (10% of reaction loaded). The pellet was resuspended in 50 μl of 6M Urea, diluted 1:1 with sample buffer, heated for 5 minutes at 95° C and electrophoresed through an SDS-PAGE gel (5% of reaction loaded).

Western blot

The protocol for the transfer of proteins from gel to transfer membrane was adapted from the manufacturer's instructions (Biorad, Inc.) Briefly, after electrophoresis, the PAGE gel was

removed from the apparatus and soaked in Transfer buffer (39 mM Glycine, 48 mM TRIS base, 0.037% SDS, 20% methanol) with two thick filter papers and an Immobilon transfer membrane (pre-soaked in methanol for 5 minutes) for 10 to 15 minutes. On a Bio-Rad semi-dry transfer apparatus, a sandwich consisting of the gel on top of the Immobilon, in between the filter papers was assembled, rolling a tube over the top to ensure that there were no air bubbles. The apparatus was assembled and subjected to 15 volts for 20 to 30 minutes (using a high current power supply that can handle up to 500 mA).

The membrane was blocked with 5% skim milk in PBST (phosphate buffered saline containing 0.05% Tween 20) at 4° C overnight or 2 hours at 37° C with shaking. The membrane was washed with PBST 3 times, 5 minutes each with shaking, and then incubated with the primary antibody (5 μg/ml in 5% skim milk in PBST) at room temperature for 60 minutes, with shaking. The membrane was again washed 3 times in PBST, then incubated with the secondary antibody (diluted to a final concentration of 2.5 μg/ml) in 5% skim milk in PBST for 60 minutes, with shaking. After washing 3 more times in PBST, the substrate was mixed (0.019 % Cobalt chloride, 0.018% hydrogen peroxide, 12.5 mg/ml diaminobenzidine in PBS) and added to the transfer membrane. The mixture was allowed to develop 1 to 5 minutes (until dark enough) and the reaction was stopped by rinsing with copious amounts of ddH_2O.

Sera banks available for testing

From the two study populations described above sera were chosen as follows:

(1) Among the children with cardiopulmonary disease, sera were chosen to include those from all study subjects who developed RSV infection and from an equal number who did not, matched for age and type of underlying disease. Although a total of 85 children followed during

the two study years developed RSV infection, sufficient sample for further testing was available for 73 only and thus the total number of sera tested was 146.

Acute sera from all previously healthy infants for whom the infecting subgroup was known were selected. Adequate samples were available for 82 with subgroup A and 127 with subgroup B infections. Selected cases were further subgrouped by the presence or absence of early hypoxia as defined in the introduction.

Paired acute-convalescent sera samples from all previously healthy children infected with a known subgroup were chosen. Sufficient sample for further testing was available for 45 subgroup A infected and 99 subgroup B infected infants and children.

Statistical data analysis

Analyses were done using the SAS statistical analysis software package. The distribution of categorical variables was compared by Fisher's exact test or chi square test, as appropriate for the study number. Normally distributed continuous variables were compared by student's t test whereas those that were not normally distributed were compared by the Wilcoxon rank sum test. For each study, antibody breakpoint titers were defined and tested by univariate analysis. All variables with a p value less than or equal to 0.1 were included in multiple logistic regression models.

Results

Analysis of pre-RSV season sera from children with cardiopulmonary disease

Titers of antibody to whole F glycoprotein, measured by ELISA correlated highly with previously determined titers of neutralizing antibody to both A2 and 18537 strains of RSV (Table 1). The R factor is the calculated correlation coefficient. Table 2 shows the distribution of risk factors, age and preseason antibody levels within the selected subsets of the compromised host population. The geometric mean titers (GMT) of neutralizing antibody to A2 and 18537 as well as the GMT of ELISA antibody to F glycoprotein among the noninfected control group were all significantly higher than those in the age-matched RSV infected group ($p<0.01$ for all, Table 2). The distribution of A2 neutralization titers were significantly different between the infected and uninfected groups for all breakpoints examined including: 10, 40, 100, 200, and 300 (Figure 3a). For the purposes of this study, a breakpoint was defined as the titer examined at which a significant observation may or may not occur. The greatest distinction between the two groups was the 1:100 breakpoint ($p<0.001$, FE, Table 3). The distribution of 18537 neutralization titers were also significantly different between the infected and uninfected groups for all the following breakpoints (Figure 3b): 10, 40, and 100. Further there was a trend for the 200 breakpoint ($0.05<p<0.1$). The best distinction between the two groups was the 1:40 breakpoint ($p=0.005$, chi square). The distribution of F ELISA titers was significantly different between the infected and uninfected groups for all breakpoints tested with the best distinction between the two groups at the 1:6000 cutoff ($p<0.001$, FE)(Figure 3c). Table 3 shows the results of the logistic regression for antibody titer breakpoints that correlated with the risk of RSV infection.

Table 1. Correlations between 3 different assays for measuring antibody to RSV in a group of compromised host sera prior to infection.

Variable(s)	R factor	P value
Subgroup A neut. titers vs. Subgroup B neut. titers.	0.803	<0.001
Subgroup B neut. titers vs. F ELISA titers.	0.722	<0.001
F ELISA titers vs. Subgroup A neut. titers.	0.780	<0.001

Table 2. Characteristics of selected RSV infected and uninfected sera from a compromised host patient population .

CHARACTERISTIC	RSV INFECTED (n=73)	RSV NOT INFECTED (n=73)	P VALUE
Demographics			
Mean Age +/- S.D.	56.8 +/- 40.0 wks	57.2 +/- 40.7 wks	ns
Age Groups:			
<27 weeks	23.3%	24.7%	ns
27-52 weeks	38.4%	35.6%	ns
1 – 2 years	24.7%	23.3%	ns
2 – 3 years	13.7%	16.4%	ns
Underlying Disease			
Congenital Hrt Disease	47.9%	52.1%	ns
Chronic Lung Disease	52.1%	47.9%	ns
Preseason Antibody Levels			
Acute Subgroup A GMT* +/- S.E.	16.02 +/- 1.2	37.10 +/- 1.2	0.002
Acute Subgroup B GMT* +/- S.E.	14.58 +/- 1.2	29.53 +/- 1.2	0.006
Acute ELISA F GMT* +/- S.E.	268.8 +/- 1.2	896.3 +/- 1.3	0.001

*GMT = geometric mean titer

Table 3. Logistic regression of the titer breakpoints which most clearly distinguished children infected with RSV and those with no known infection.

Breakpoint	% Infected Above Breakpoint	% Uninfected Above Breakpoint	Univariate p value*	Odds Ratio (95% confidence)
A2 neut ≥1:100	9.9%	33.8%	<0.001	0.3 (0.1 – 0.9): p=0.04
18537 neut ≥1:100	9.9%	25.4%	0.026	0.6 (0.2 – 2.0)
F ELISA ≥1:4000	9.6%	27.4%	<0.01	0.5 (0.2 – 1.7)
A2 neut ≥1:200	4.2%	19.7%	0.008	0.26 (0.06 – 1.2)
18537 neut ≥1:200	4.2%	12.7%	0.1	1.4 (0.2 – 8.7)
F ELISA ≥1:6000	4.1%	24.7%	<0.001	0.11 (.02 – 0.56): p=0.008

* all titers by univariate analysis tested by Fisher Exact (FE)

Table 4. Characteristics of patient population for whom single acute sera was collected.

CHARACTERISTIC	SUBGROUP A INFECTION		P Value	SUBGROUP B INFECTION		P VALUE
	Hypoxic (n=26)	Not Hypoxic (n=56)		Hypoxic (n=68)	Not Hypoxic (n=149)	
Demographics						
Age						
Mean +/- S.D. (weeks)	14.08 +/- 20.7	29.36 +/- 27.8	0.01	21.91 +/- 20.5	29.48 +/- 24.9	0.03
% <13 weeks	81%	38%	0.001 (F.E)	46%	30%	0.02 (chi)
% <37 Weeks gestation	8%	14%	ns	22%	15%	ns
Gender (% male)	54%	57%	ns	66%	68%	ns
Ethnicity (% Aboriginal)	38%	16%	≤0.05	45%	11%	≤0.01
Acute Serum Antibody (GMT +/- S.E.)						
A2 neut. GMT +/- S.E.	59.71 +/- 1.3	26.78 +/- 1.2	<0.05	33.90 +/- 1.2	23.69 +/- 1.1	0.06
18537 neut. GMT +/- S.E.	41.06 +/- 1.3	29.36 +/- 1.2	ns	42.02 +/- 1.2	27.63 +/- 1.1	0.06
F ELISA GMT +/- S.E.	1101.5 +/- 1.4	406.2 +/- 1.2	<0.05	330.5 +/- 1.2	516.4 +/- 1.1	0.06

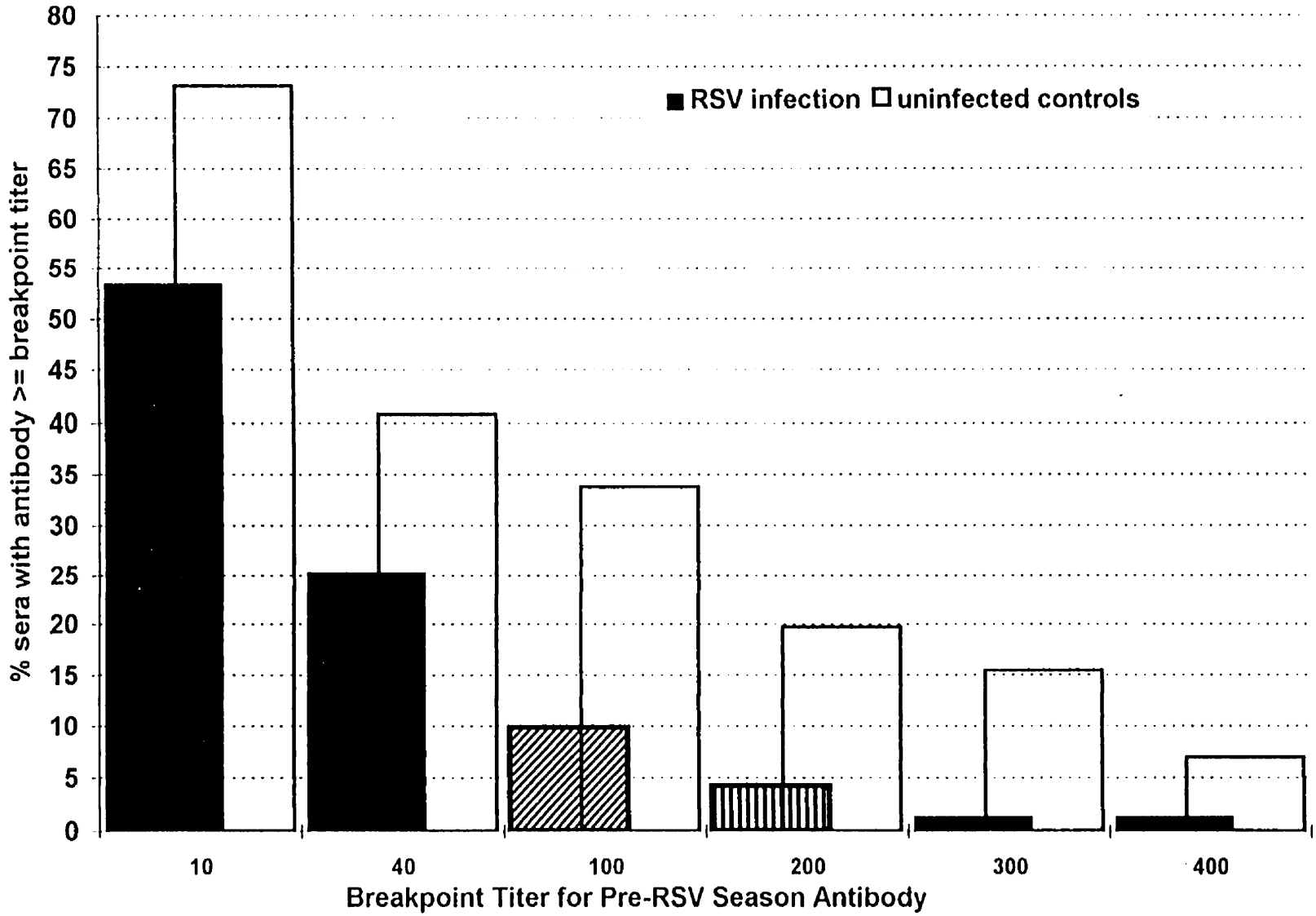

Figure 3a. Correlation between RSV A2 neutralizing antibody and subsequent RSV infection among infants with cardiopulmonary disease. Sera from 73 RSV-infected and 73 non-infected infants with cardiopulmonary disease (matched for age and underlying disease) were tested by neutralization assay against strain A2 of RSV and the distribution of titers were examined for all breakpoints. Diagonal hatched bar: breakpoints exceeded by fewer than 10% of the infected group. Vertical hatched bar: breakpoints exceed by fewer than 5% of the infected group.

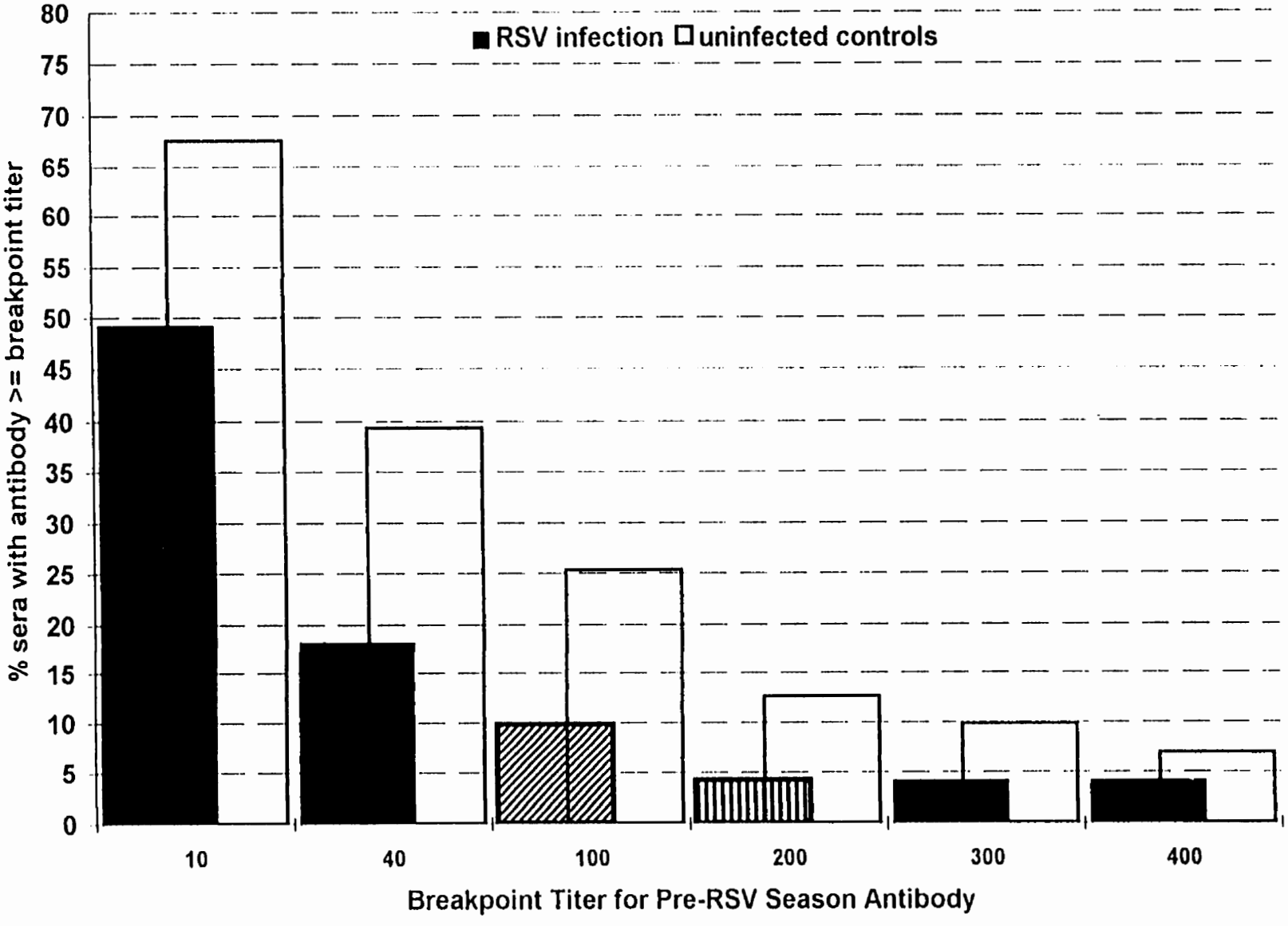

Figure 3b. Correlation between RSV 18537 neutralizing antibody and subsequent RSV infection among infants with cardiopulmonary disease. Sera from 73 RSV-infected and 73 non-infected infants with cardiopulmonary disease (matched for age and underlying disease) were tested by neutralization assay against strain 18537 of RSV and the distribution of titers were examined for all breakpoints. Diagonal hatched bar: breakpoints exceeded by fewer than 10% of the infected group. Vertical hatched bar: breakpoints exceed by fewer than 5% of the infected group.

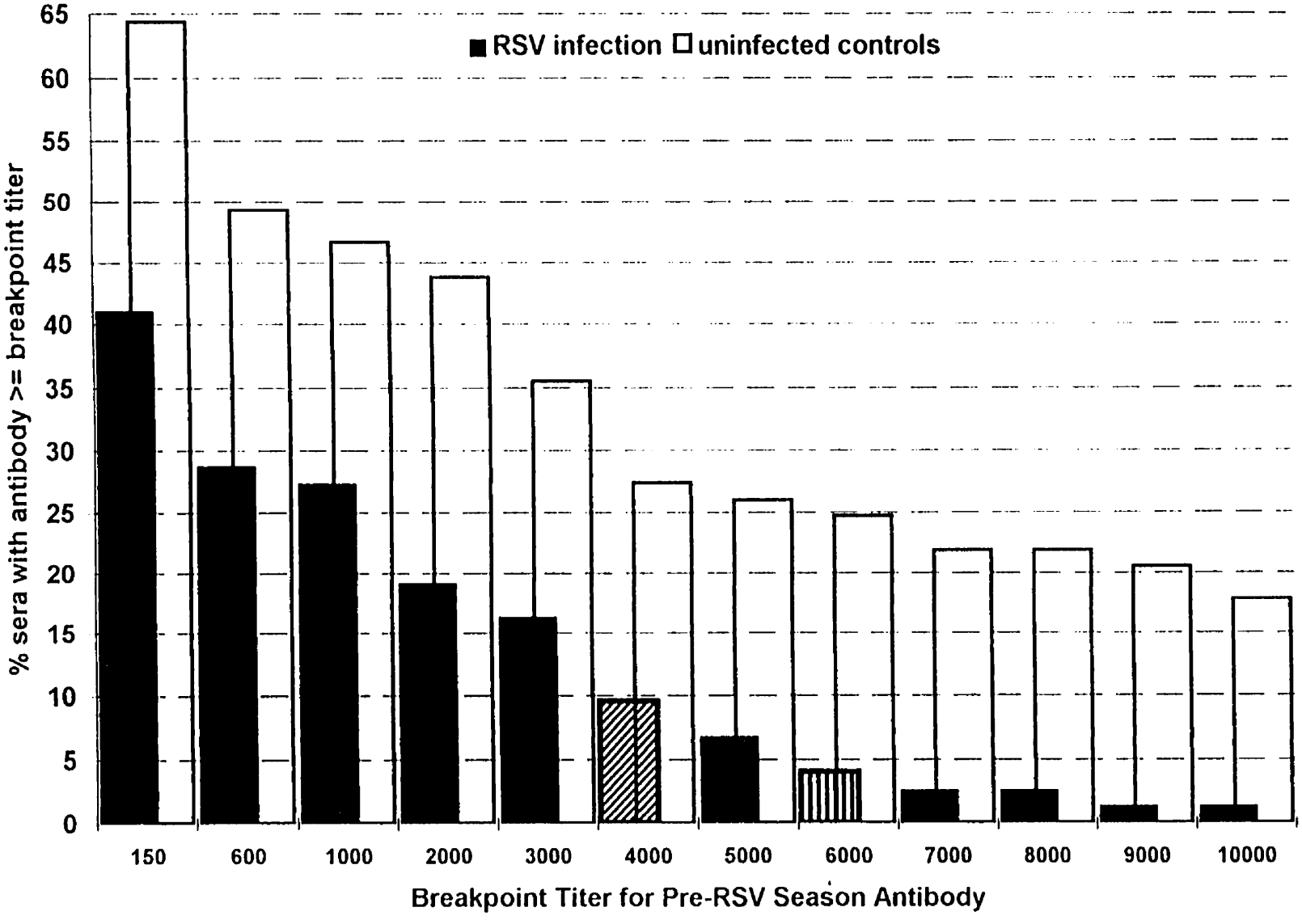

Figure 3c. Correlation between RSV F glycoprotein antibody and subsequent RSV infection among infants with cardiopulmonary disease. Sera from 73 RSV-infected and 73 non-infected infants with cardiopulmonary disease (matched for age and underlying disease) were tested by ELISA assay using the F glycoprotein as the antigen and the distribution of titers were examined for all breakpoints. Diagonal hatched bar: breakpoints exceeded by fewer than 10% of the infected group. Vertical hatched bar: breakpoints exceed by fewer than 5% of the infected group.

For breakpoints exceeded by fewer than 10% of the infected group, only the A2 neutralization breakpoint of greater than or equal to 1:100 was significant (odds ratio (OR) = 0.3, 95% confidence intervals (CI) 0.1 – 0.9). For breakpoints exceeded by fewer than 5% of the infected group, only the F ELISA breakpoint of greater than or equal to 1:6000 was significant (OR = 0.1, 95% CI 0.02 – 0.56).

Analysis of acute sera from previously healthy infants hospitalized for RSV infection

Table 4 shows characteristics of infants hospitalized for RSV for whom acute sera was available for testing divided according to infecting subgroup and presence or absence of early hypoxia. Significant differences were observed ($p<0.05$) between hypoxic and non-hypoxic patients in terms of age, GMT's of A2 and 18537 neutralizing antibody, F protein ELISA antibody as well as aboriginal race. Similar trends for A and B infected infants were observed with one exception: F ELISA antibody was significantly lower among B infected hypoxic infants, an opposite trend to that observed for all other GMT comparisons. Young age was significantly related to hypoxia for both subgroup A and subgroup B infected infants (stronger trend for subgroup A infection). Given significant differences in GMT, a breakpoint titer was examined for neutralizing and ELISA antibody that significantly distinguished between hypoxic and non-hypoxic subgroups (Figure 4, a-c). By univariate analysis for subgroup A infection the breakpoint for both A2 and 18537 was greater than or equal to 40, and for F ELISA was greater than or equal to 1000, whereas for subgroup B infections the breakpoint for A2 and 18537 was greater than or equal to 10, and for F ELISA was greater than or equal to 1000.

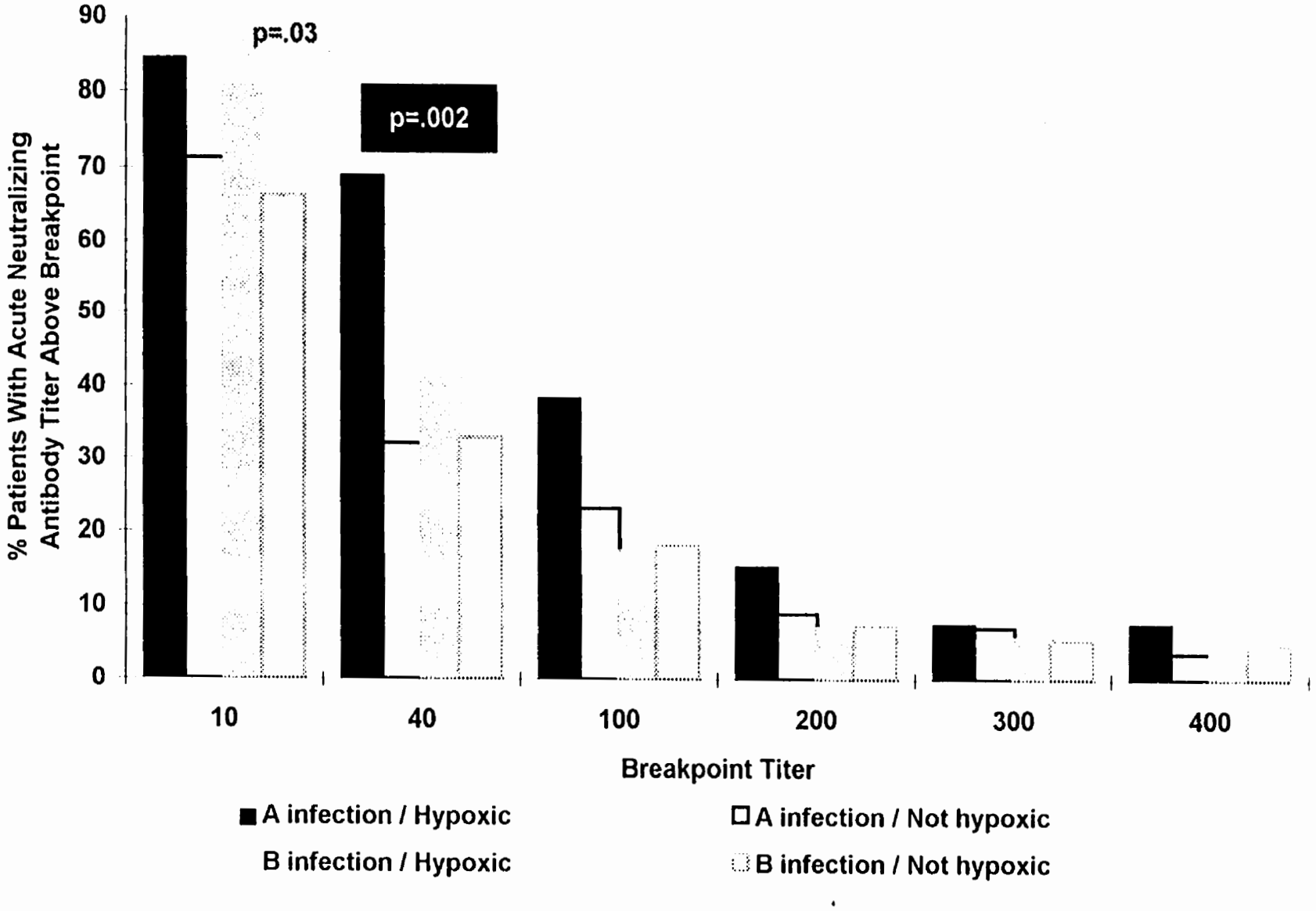

Figure 4a. Association between acute RSV subgroup A neutralizing antibody titer and hypoxia documented within 48 hours of hospital admission. Acute sera samples from previously healthy infants for whom infecting subgroup was known (82 infected with subgroup A, 127 infected with subgroup B) were analyzed by neutralization assay against the A2 strain of RSV. Breakpoint titers were examined that significantly distinguished between hypoxic and non-hypoxic subgroups. For each group of 4 histograms, the bars are labelled as follows: Bar #1, subgroup A infection / hypoxic. Bar #2, subgroup A infection / not hypoxic. Bar #3, subgroup B infection / hypoxic. Bar #4, subgroup B infection / not hypoxic.

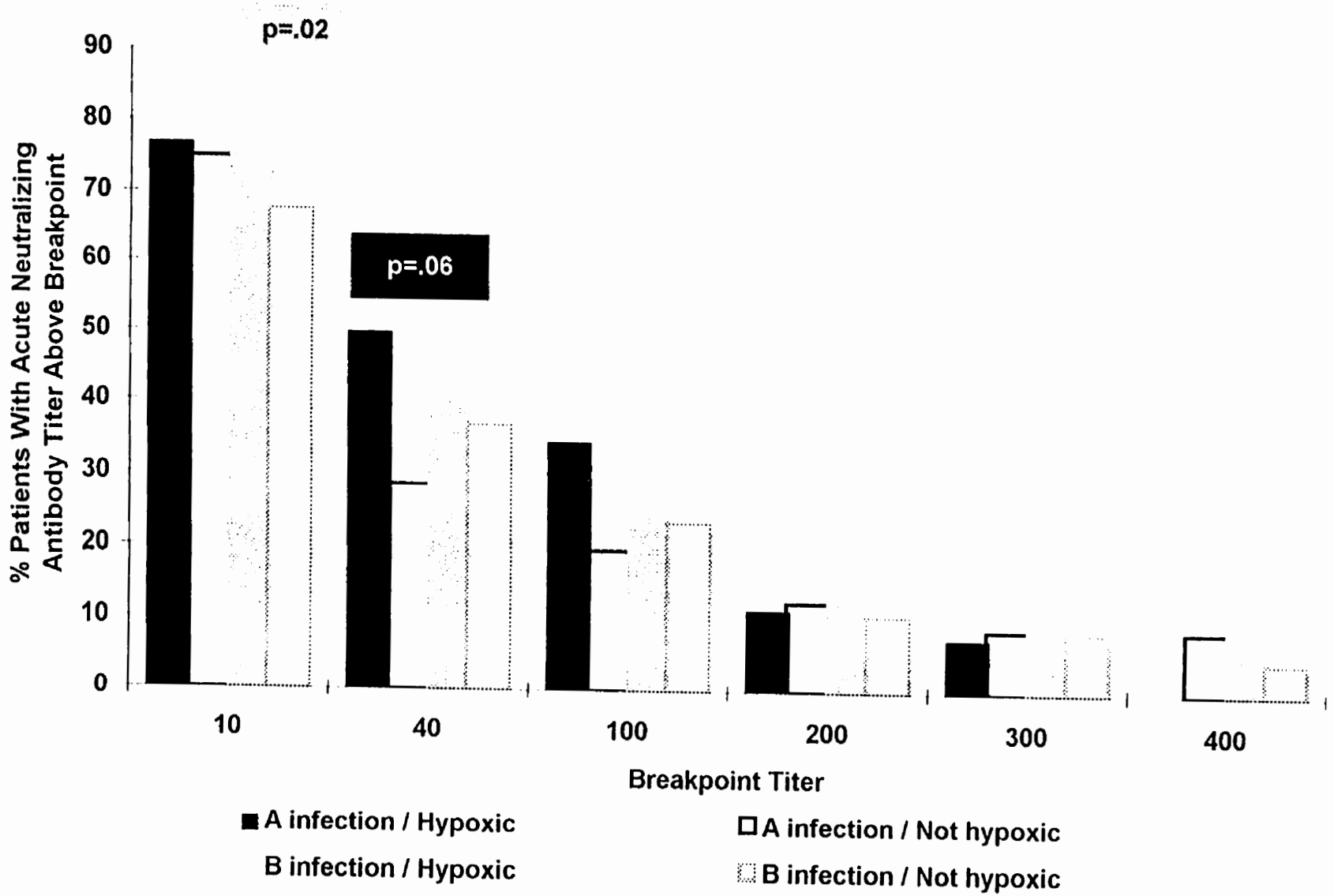

Figure 4b. Association between acute RSV subgroup B neutralizing antibody titer and hypoxia documented within 48 hours of hospital admission. Acute sera samples from previously healthy infants for whom infecting subgroup was known (82 infected with subgroup A, 127 infected with subgroup B) were analyzed by neutralization assay against the 18537 strain of RSV. Breakpoint titers were examined that significantly distinguished between hypoxic and non-hypoxic subgroups. For each group of 4 histograms, the bars are labelled as follows: Bar #1, subgroup A infection / hypoxic. Bar #2, subgroup A infection / not hypoxic. Bar #3, subgroup B infection / hypoxic. Bar #4, subgroup B infection / not hypoxic.

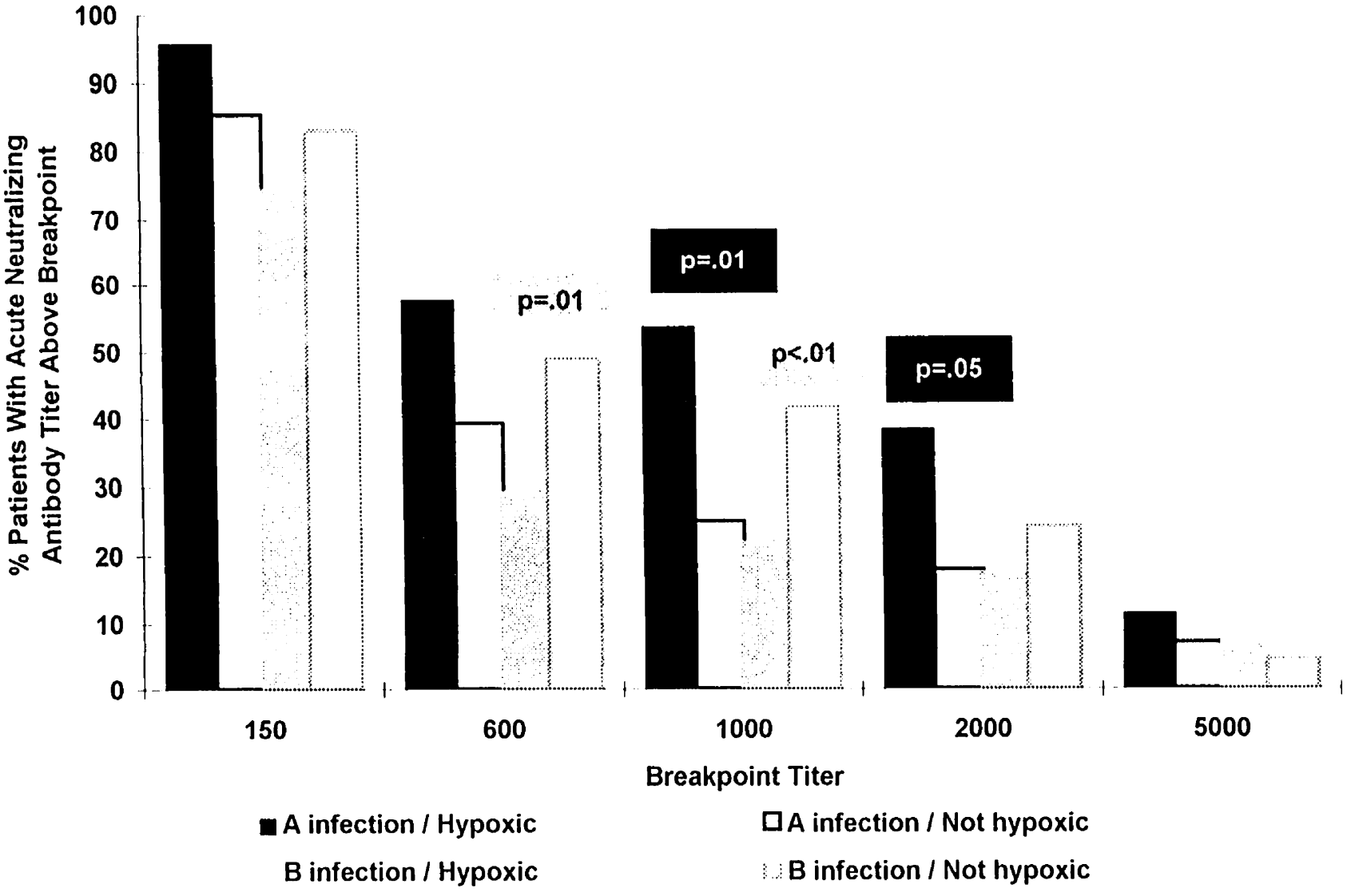

Figure 4c. Association between acute RSV F glycoprotein ELISA antibody titer and hypoxia documented within 48 hours of hospital admission. Acute sera samples from previously healthy infants for whom infecting subgroup was known (82 infected with subgroup A, 127 infected with subgroup B) were analyzed by ELISA using the F glycoprotein as the antigen. Breakpoint titers were examined that significantly distinguished between hypoxic and non-hypoxic subgroups. For each group of 4 histograms, the bars are labelled as follows: Bar #1, subgroup A infection / hypoxic. Bar #2, subgroup A infection / not hypoxic. Bar #3, subgroup B infection / hypoxic. Bar #4, subgroup B infection / not hypoxic.

By logistic regression (Table 5), for A infections, age less than 13 weeks was the only independently significant factor associated with a 5 fold higher likelihood of hypoxia, whereas for B infected infants age was significantly associated with a two fold increase in likelihood of hypoxia ($p<0.05$). Conversely, an F ELISA titer of greater than or equal to 1:1000 was associated with a 5 fold decrease in the likelihood of hypoxia for B infected children only ($p<0.001$).

Analysis of convalescent paired sera from previously healthy infants and children hospitalized with RSV infection

The demographics, illness characteristics and acute antibody titers for the subgroup A and B infected patient population are shown in table 6, divided according to age (less than or greater than 6 months). For both young and older age groups infected by subgroup A or B, the distribution of demographic variables, including gender, gestation period, and ethnicity was not significantly different. However, infants less than 6 months were significantly more likely to have: (1) severe disease as manifested by hypoxia, ventilation and admission to intensive care and (2) measurable amounts of neutralizing and F ELISA antibody, most likely maternal in origin. Tables 7a and 7b illustrate the age-related homologous and heterologous convalescent neutralizing antibody response among infants infected with subgroup A or subgroup B, respectively. Three similar trends were observed among both A and B infected cases: (1) infants aged more than 26 weeks were significantly more likely than their younger counterparts to seroconvert or have a 4 fold or higher rise in both homologous and heterologous neutralizing antibody, (2) the acute to convalescent rise in both homologous and heterologous neutralizing antibody as measured by GMT was significantly higher among older infants and (3) the homologous neutralizing antibody response exceeded the heterologous neutralizing response for

Table 5. Independent variables associated with severe RSV disease defined as hypoxia occurring within 48 hours of admission to hospital.

Infecting Subgroup	Variable	Odds Ratio (95% Confidence)	p value
A	Age <13 weeks	4.9 (1.4 - 17.6)	0.01
B	Age <13 weeks	2.1 (1.02 – 4.4)	0.04
B	F ELISA titer ≥1:1000	0.2 (0.1 – 0.5)	0.0003

Table 6. Characteristics of patient population infected with RSV, for whom paired sera was collected.

CHARACTERISTIC	SUBGROUP A INFECTION		P value	SUBGROUP B INFECTION		P value
	<27 weeks (n=27)	≥27 weeks (n=18)		<27 weeks (n=58)	≥27 weeks (n=41)	
Demographics						
Age (weeks) median +/- S.D (and range)	8.7 +/- 6.2 (1-25)	44.2 +/- 20.2 (27-89)	ns	12.6 +/- 7.5 (1-26)	49.3 +/- 19.4 (27-104)	ns
Gender (% male)	55.6 %	44.4%	ns	74.1%	63.4%	ns
Gestation (% term)	92.6%	77.8%	ns	75.9%	85.4%	ns
Ethnicity (% Caucasian)	63.0%	50.0%	ns	77.6%	73.2%	ns
Ethnicity (% Aboriginal)	7.4%	5.6%	ns	3.5%	9.8%	ns
Ethnicity (% Other)	29.6%	44.4%	ns	18.2%	17.1%	ns
Disease Severity						
% hypoxia	37.0%	5.6%	ns	81.0%	22.0%	ns
% ventilated	11.1%	0.0%	ns	10.3%	2.4%	ns
% admitted to ICU	18.5%	5.6%	ns	24.1%	4.9%	ns
Prior Antibody Levels						
Subgroup A (% >1:40)	48.1%	16.7%	0.06	36.2%	12.2%	0.06
Subgroup B (% >1:40)	44.4%	11.1%	<0.05	39.7%	17.1%	<0.05
F ELISA (% >1:150)	77.8%	27.8%	<0.05	65.5%	29.3%	<0.05

TABLE 7a. Neutralization titers among infants and children infected with RSV Subgroup A.

Patient Age	No. Tested	Homologous Response			Heterologous Response		
		Geometric Mean Titer		Response *	Geometric Mean Titer		Response *
		Acute (A)	Convalescent (C)		Acute (A)	Convalescent (C)	
<27 weeks	27	44.7 +/- 1.3	68.2 +/- 1.2	19%	50.1 +/- 1.2	44.3 +/- 1.2	7%
≥27 weeks	18	14.7 +/- 1.4	251.2 +/- 1.4	94%	12.5 +/- 1.4	102.8 +/- 1.3	78%
<27 vs ≥27 weeks		A to C rise in GMT (Wilc) p <0.001			A to C rise in GMT (Wilc) p <0.001		

* Response is defined as a 4 fold rise in titer and/or seroconversion (from non-detectable to detectable levels of antibody)

TABLE 7b. Neutralization titers among infants and children infected with RSV Subgroup B.

Patient Age	No. Tested	Homologous Response			Heterologous Response		
		Geometric Mean Titer		Response *	Geometric Mean Titer		Response *
		Acute (A)	Convalescent (C)		Acute (A)	Convalescent (C)	
<27 weeks	58	31.8 +/- 1.2	41.9 +/- 1.1	15%	33.6 +/- 1.2	88.3 +/- 1.1	33%
≥27 weeks	41	11.4 +/- 1.2	100.3 +/- 1.2	76%	12.1 +/- 1.2	258.8 +/- 1.2	88%
<27 vs ≥27 weeks		A to C rise in GMT (Wilc) p <0.001			A to C rise in GMT (Wilc) p <0.001		

* Response is defined as a 4 fold rise in titer and/or seroconversion (from non-detectable to detectable levels of antibody)

TABLE 7c. F ELISA IgG titers of infants and children infected with RSV subgroup A.

Patient Age	No. Tested	Geometric Mean Titers +/- S.E.		Response*
		Acute (A)	Convalescent (C)	
<27 weeks	27	609.5 +/- 1.4	1008.3 +/- 1.3	26%
≥27 weeks	18	194.1 +/- 1.5	7035.1 +/- 1.5	89%
<27 vs ≥27 weeks		A to C rise in GMT (Wilc) p <0.001		

* Response is defined as a 4 fold rise in titer and/or seroconversion (from non-detectable to detectable levels of antibody)

both age groups regardless of infecting subgroup. Among infants less than 6 months of age, the only significant convalescent antibody response observed was for neutralizing antibody to B among subgroup B infected infants.

Tables 7c and 7d illustrate the F ELISA antibody response among A and B infected infants respectively. Among A infected patients there was a significant age-related difference in response, similar to that observed for neutralizing antibody. Specifically, infants aged more than 6 months had significantly higher rates of seroconversion and significantly higher acute to convalescent rises in GMT. A similar observation was observed for subgroup B infected cases. however the differences were less marked and did not quite reach significance for either seroconversion or rise in GMT. Infants less than 27 weeks and infected with subgroup A were less likely to have a significant rise in F ELISA antibody titer than similarly aged patients infected with subgroup B (26% vs 55% respectively). The acute to convalescent rise in titer was significantly less for A vs B infected infants less than 27 weeks of age (2 fold versus 5 fold respectively). An opposite trend was observed for infants aged greater than or equal to 27 weeks in that the F response following subgroup A infections was stronger than that following B infections both in terms of percent making a significant response (89% vs 73%) and fold change in GMT (36 fold vs 17 fold).

Acute titers to F glycoprotein were similar among infants aged greater than or equal to 27 weeks infected with either subgroup A (GMT=194) or B (GMT=210) and among B infected infants aged less than 27 weeks (GMT=311) whereas A infected infants less than 27 weeks had higher titers (GMT=609).

Table 8 shows the results of the logistic regression for factors associated with a homologous neutralizing antibody response. For both A and B infections, young age was

TABLE 7d. F ELISA IgG titers of infants and children infected with RSV subgroup B.

Patient Age	No. Tested	Geometric Mean Titers +/- S.E.		Response*
		Acute (A)	Convalescent (C)	
<27 weeks	58	311.3 +/- 1.1	1579.9 +/- 1.2	55%
≥27 weeks	41	210.2 +/- 1.3	3591.9 +/- 1.3	73%
<27 vs ≥27 weeks		A to C rise in GMT (Wilc) p =0.09		

* Response is defined as a 4 fold rise in titer and/or seroconversion (from non-detectable to detectable levels of antibody)

Table 8. Logistic regression for variables associated with making a positive homologous convalescent antibody response.

Variable	Age (≥27 weeks)		Prior B Antibody (<1:40)	
	Odds ratio (95% confidence intervals)	P value	Odds ratio (95% confidence intervals)	P value
Both subgroup infections	27.98 (7.28 – 107.5)	<0.001	16.09 (3.76 – 68.8)	<0.001
Subgroup A Infection	94.107 (6.75 – 1312.4)	<0.001	24.128 (0.70 – 835.2)	ns
Subgroup B Infection	21.759 (4.36 – 108.6)	<0.001	21.805 (3.83 – 124.2)	<0.001

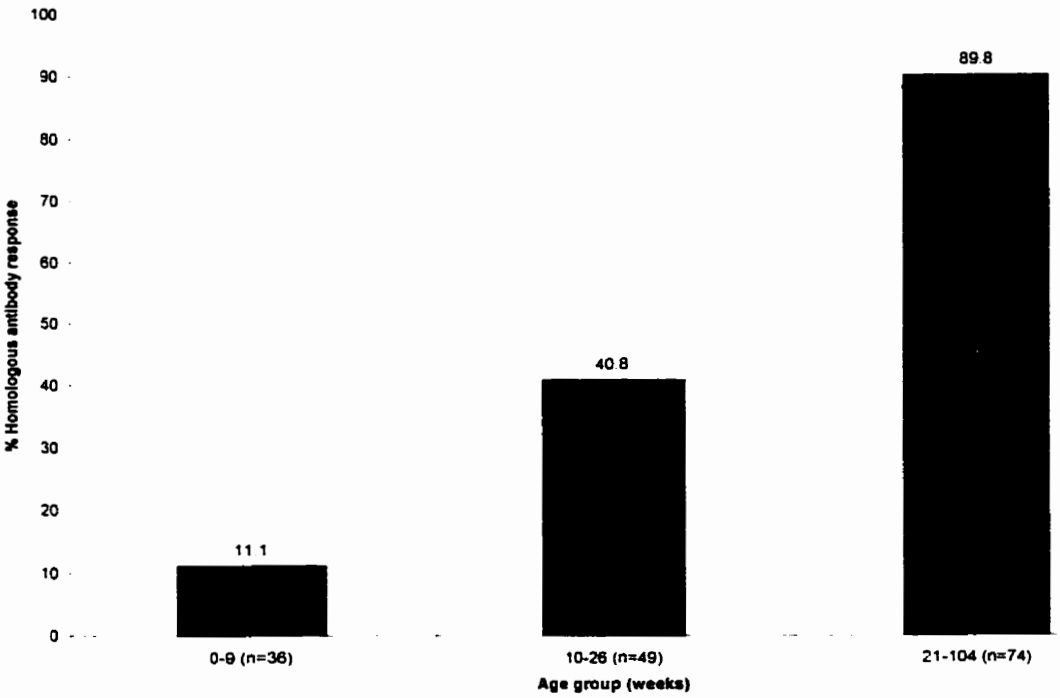

Figure 5. Effect of age on the homologous response. Paired acute-convalescent sera were collected from infants hospitalized with RSV infection and tested by neutralization assay. Three different age groups are shown. An antibody response was measured as a 4-fold or greater rise in titer or seroconversion.

significantly associated with a poor convalescent antibody response. A neutralizing antibody titer of >1:40 against subgroups B was significantly associated with a poor homologous response for the B infected infants only.

Reverse Transcriptase and PCR of the F protein

The PCR products (fragments #1, #2 and #3) were predicted to exhibit base pair lengths of 891, 336 and 645 respectively, confirmed by agarose gel electrophoresis (figure 6). Although the amplification of the 18537 strain is shown, identical results were obtained using the A2 strain.

DNA Enzyme Digestion and Purification

In preparation for cloning into pBluescript, "2FB" (the 2nd fragment from the F protein of the B strain; 18537) was digested with the restriction endonucleases BAMHI and BglII, and pBluescript was digested with BAMHI and treated with Calf Intestinal Phosphatase (CIP) to reduce cloning background (figure 7A). After being ligated, transformed into XL-I Blue® bacterial cells, and plated on LB-Amp-X-Gal plates, greater than 90% of the colonies were white (i.e. the β-galactosidase gene was interrupted due to the target DNA being inserted into the multiple cloning site, inhibiting the cell from converting the substrate X-galactose into a blue product). After the alkali-lysis miniprep procedure was performed, presence of the 2FB insert was confirmed by release of target DNA via restriction enzyme digestion (figure 7B).

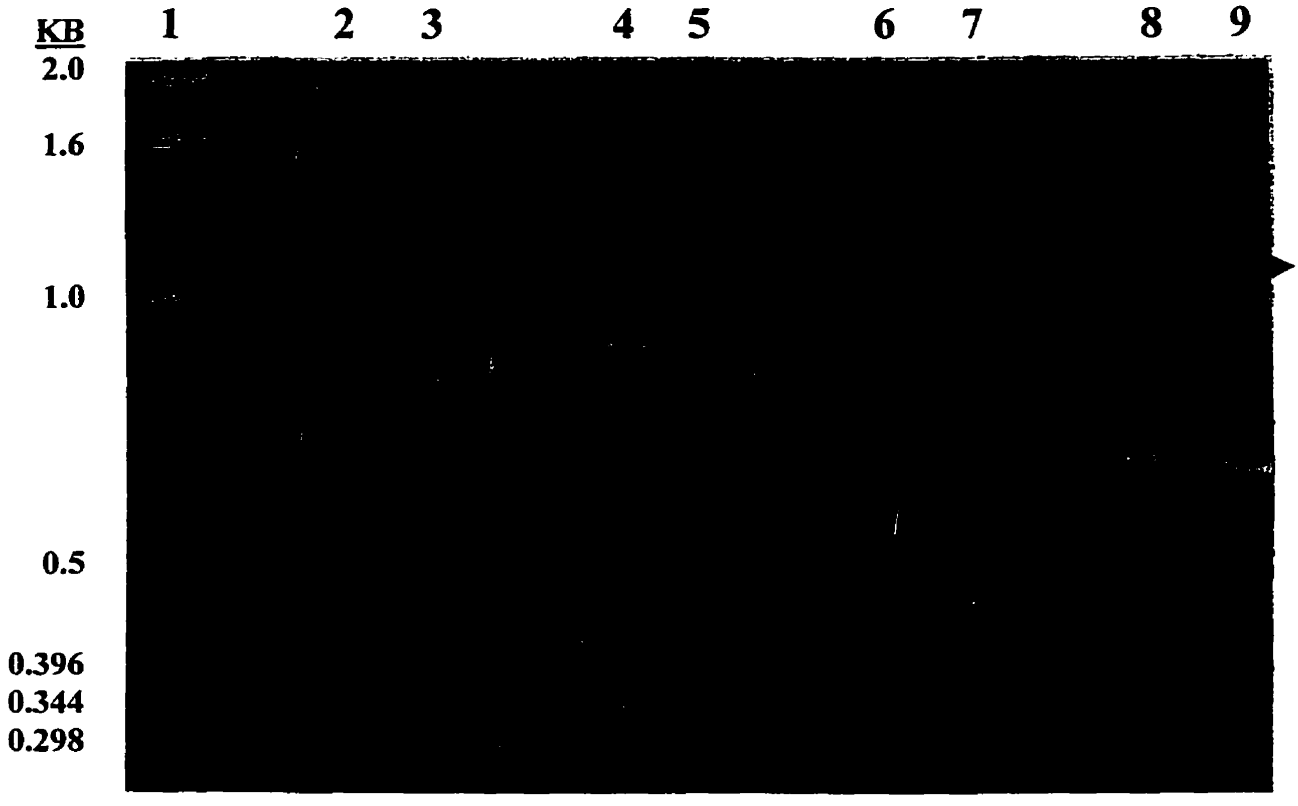

Figure 6. Agarose gel electrophoresis of PCR products amplified from the F protein of the 18537 strain of Respiratory Syncytial Virus. The various PCR products (5% of each reaction per lane) were separated on a 1% agarose gel, stained with ethidium bromide and visualized under UV light. Lane 1, 1 Kilobase standard base pair ladder. Lanes 2, 3, - cDNA control. Lanes 4, 5, 1st fragment (1FB). Lanes 6,7, 2nd fragment (2FB). Lanes 8, 9, 3rd fragment (3FB).

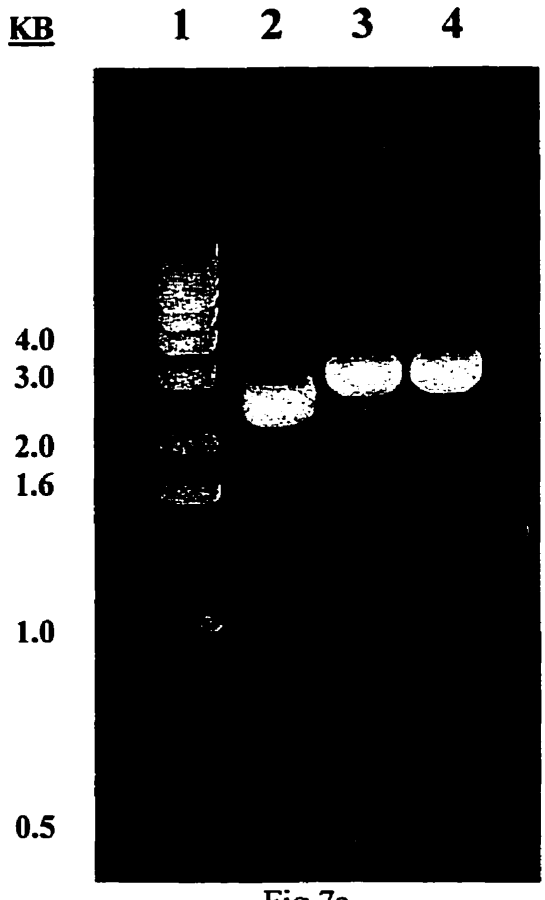

Fig 7a

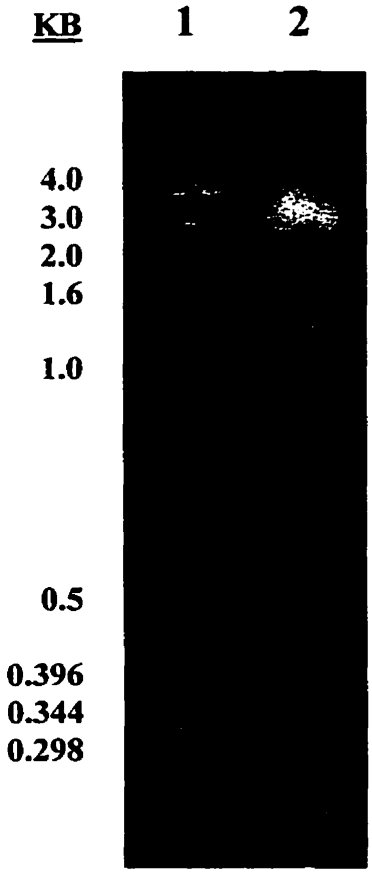

Fig 7b

Figure 7a. Electrophoretic analysis to determine successful digestion of plasmid. pBluescript was digested with BAMHI for 2 hours at 37° C, electrophoresed through a 1% agarose gel, stained with ethidium bromide and visualized under UV light. Lane 1, 1 Kilobase standard base pair ladder. Lane 2, Uncut pBluescript control. Lanes 3, 4, pBluescript digested with BAMHI.

Figure 7b. Confirmation of ligation of 2FB DNA into pBluescript via restriction enzyme analysis. The 2nd F protein fragment from the B strain of RSV was released from pBluescript by digesting with BAMHI and EcoRI for 2 hours at 37° C, analyzed on a 1% agarose gel stained with ethidium bromide and visualized under UV light (uncut control not shown). Lane 1, 1 Kilobase standard base pair ladder. Lane 2, Top band, pBluescript, bottom band, released 2FB DNA fragment.

Expression of the 2FB Protein

After cloning into pBluescript, 2FB was subcloned into the glutahione-S-transferase fusion vector pGEX2T (to give pGEX2T/2FB) by excising the fragment with the restriction enzymes BAMHI and EcoRI, and ligating it into a pGEX2T plasmid that had also been treated with BAMHI and EcoRI (figure 8a). Clones #1, #2 and #3 (lanes 3, 4 and 5 respectively, figure 8A) were induced in an attempt to express the fusion protein (figure 8b). To test if the expressed fusion protein was soluble, a solubility test was performed (figure 8c), and it was found that the GST/2FB protein was insoluble. The insoluble fraction contained the band at 40 KD , which was absent in the lane where the soluble fraction was run.

The Maltose Binding Protein (MBP) fusion expression system was next attempted due to its flexibility of allowing for expression in the cell's periplasm, possibly making some proteins soluble. The 2FB fragment was subcloned from pBluescript/2FB into the pMalC and pMalP vectors using the restriction enzymes XBAI and PstI. The pMalC vector differs from the pMalP vector in that pMalP has the membrane insertion signal, thus allowing the fusion protein to be expressed in the periplasm. 2 clones from each were induced and tested for solubility (figures 8d and 8e). The pMalP vector (expressed in the periplasm) was found to express soluble fusion protein, while the fusion protein produced in the cytoplasm was insoluble (pMalC/2FB). This difference in solubility was most likely due to the expression location difference between the cytoplasm and the periplasm. In figure 8e, the maltose binding protein expressed from the pMalP vector is migrating at it's predicted molecular weight of 40 KD (lanes 5,6) and the MBP/2FB in lanes 13 and 14 shows an expected increase in molecular weight of 13 KD (equal to the predicted molecular weight of 2FB). This implies that the maltose binding protein has 2FB fused to it, but further confirmation was necessary, and a Western immunoblot was performed.

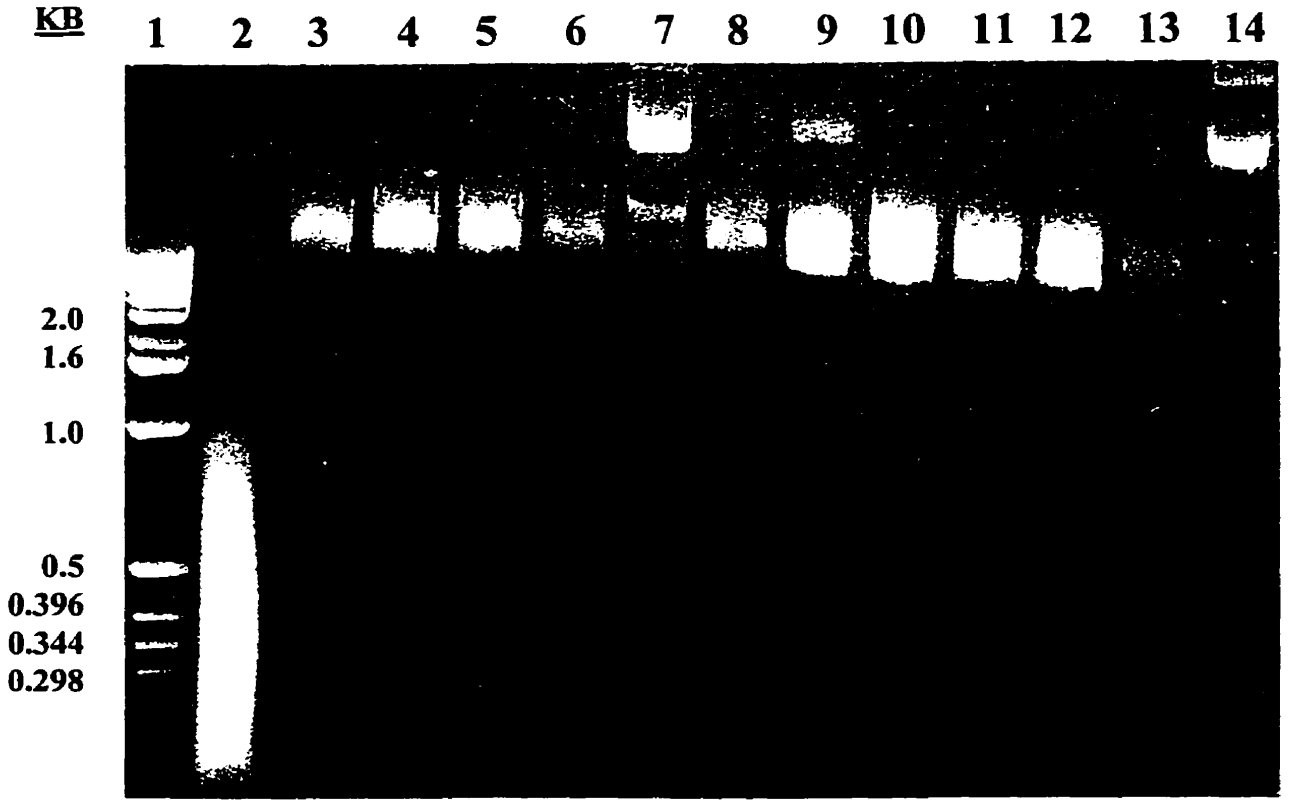

Figure 8a. Subcloning of the 2FB fragment into the pGEX2T vector. The 2FB DNA fragment was subcloned from pBluescript to pGEX2T. 12 recombinant bacterial clones were amplified and subjected to alkali lysis, digested with BAMHI and EcoRI, electrophoresed through a 1% agarose gel stained with ethidium bromide and visualized under UV light. Lane 1, 1 Kilobase standard base pair ladder. Lane 2, Uncut control. Lanes 3 through 14, random recombinant bacterial clones digested with BAMHI and EcoRI. Upper bands: pGEX2T plasmid, lower bands: recovered 2FB DNA fragment.

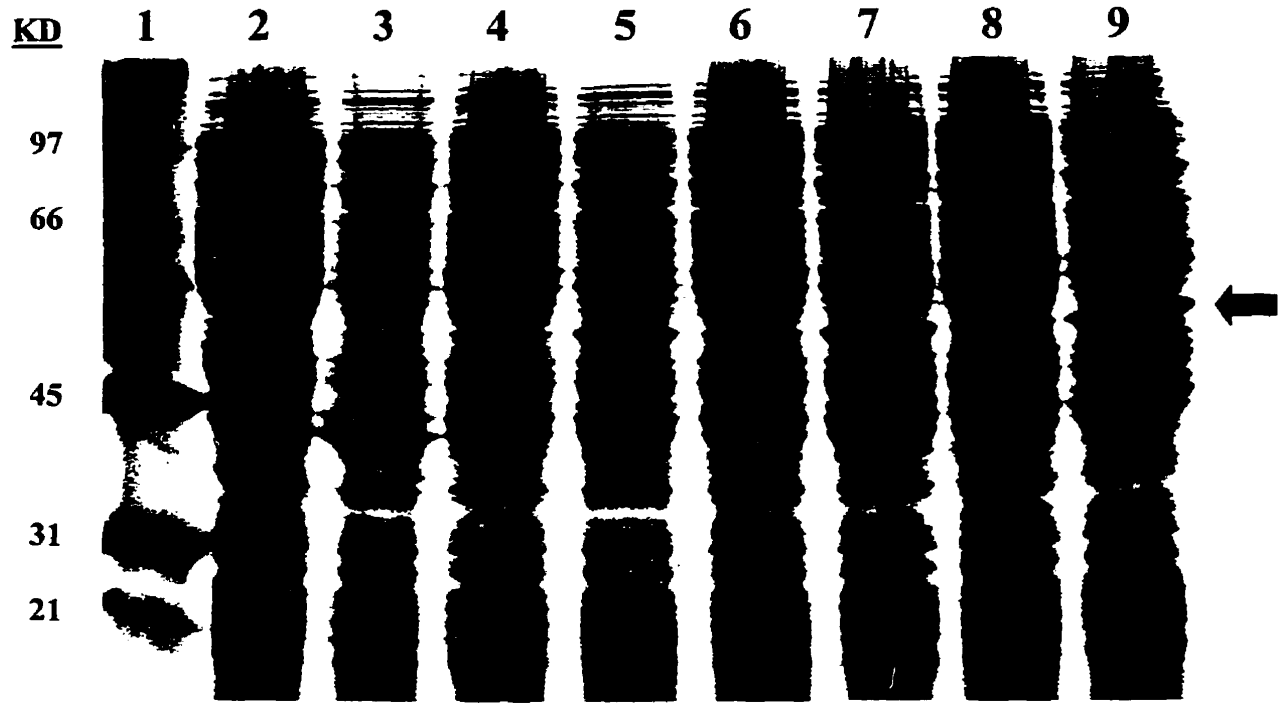

Figure 8b. Expression of Glutathione-s-transferase 2FB fusion protein in XL1Blue cells. 3 recombinant clones were induced with 0.1 mM IPTG for 1 hour at 37° C with shaking, and analyzed via SDS-PAGE on a 10% polyacrylamide gel (7.5% of each culture loaded, stained with Coomassie Blue). Lane 1: Low molecular weight standard. Lane 2, un-induced pGEX2T. Lane 3, induced pGEX2T. Lane 4, Clone #1, un-induced. Lane 5, Clone #1, induced. Lane 6, Clone #2, un-induced. Lane 7, Clone #2, induced. Lane 8, Clone #3, un-induced. Lane 9, Clone #3, induced. Note the difference in migration between GST (27.5 KD) and the GST/2FB fusion (27.5 KD + 13 KD = 40 KD). Arrow: Position of expressed GST/2FB fusion recombinant protein.

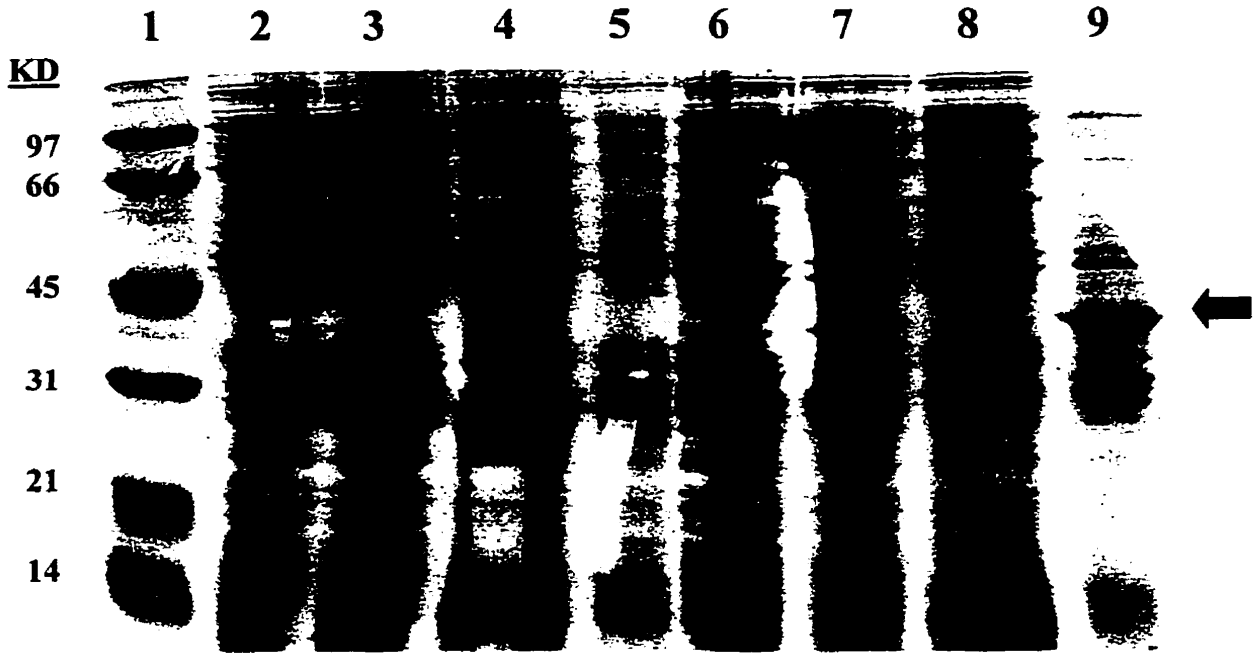

Figure 8c. Determination of solubility of pGEX2T/2FB. pGEX2T/2FB Clone #3 was tested for solubility by separating the soluble and insoluble portions and electrophoresing them through a 10% SDS-PAGE gel stained with Coomassie Blue. Lane 1: Low molecular weight standard. Lane 2, un-induced pGEX2T. Lane 3, pGEX2T, induced (whole extract). Lane 4, pGEX2T, induced (soluble fraction). Lane 5, pGEX2T, induced (insoluble fraction). Lane 6, pGEX2T/2FB, uninduced. Lane 7, pGEX2T/2FB, induced (whole extract). Lane 8, pGEX2T/2FB, induced (soluble fraction). Lane 9, pGEX2T/2FB, induced (insoluble fraction). Arrow: Position of expressed and insoluble GST/2FB fusion protein. Note: opaque areas are due to wrinkles in the cellophane gel sandwich.

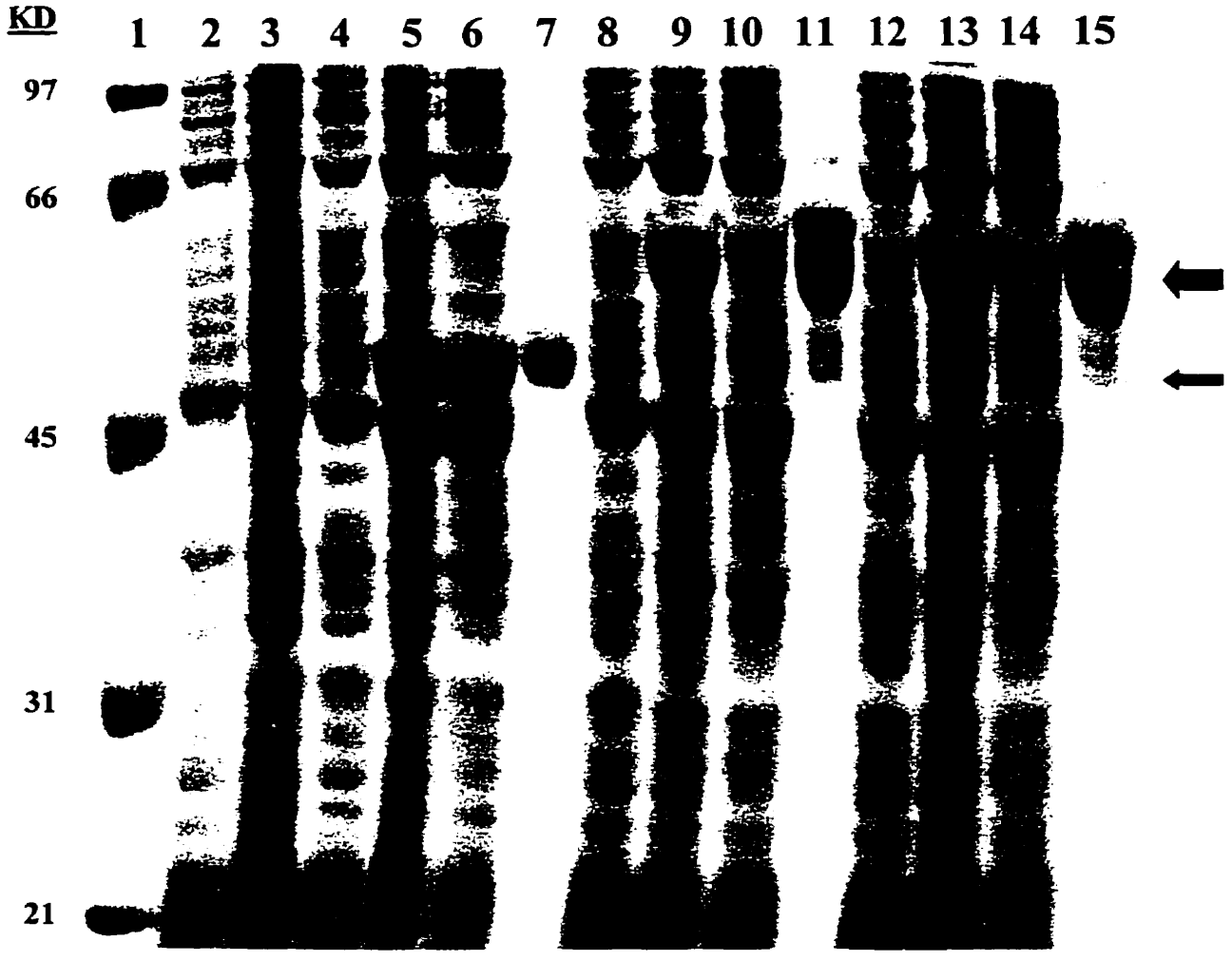

Figure 8d. Expression and determination of solubility of pMalC/2FB. pMalC/2FB clones #2 and #3 were tested for solubility by separating the soluble and insoluble portions and electrophoresing them through a 10% SDS-PAGE gel stained with Coomassie Blue. Lane 1: Low molecular weight standard. Lane 2, XL1Blue cell control, un-induced. Lane 3, XL1Blue cell control, induced. Lane 4, pMalC vector, un-induced. Lane 5, pMalC, induced (whole extract). Lane 6, pMalC, induced (soluble fraction). Lane 7, pmalC, induced (insoluble fraction). Lane 8. pMalC/2FB Clone #2, un-induced. Lane 9, pMalC/2FB Clone #2, induced (whole extract). Lane 10, pMalC/2FB Clone #2, induced (soluble fraction). Lane 11, pMalC/2FB Clone #2, induced (insoluble fraction). Lane 12, pMalC/2FB Clone #3, uninduced. Lane 13, pMalC/2FB Clone #3, induced (whole extract). Lane 14, pMalC/2FB Clone #3, induced (soluble fraction). Lane 15, pMalC/2FB Clone #3, induced (insoluble fraction). Small arrow: Position of expressed MBP. Large arrow: Position of expressed MBP/2FB fusion recombinant protein.

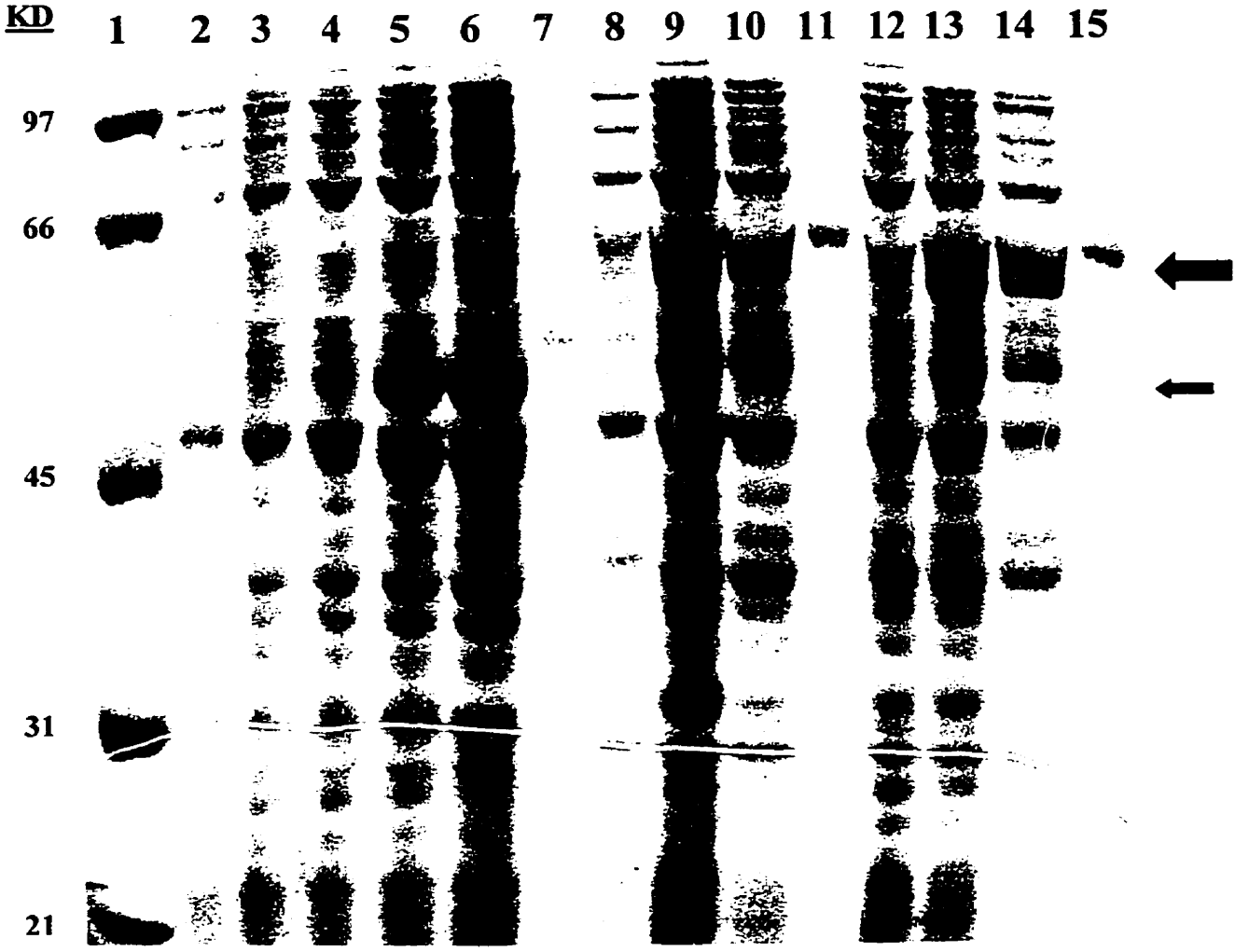

Figure 8e. Expression and determination of solubility of pMalP/2FB. pMalP/2FB clones #2 and #3 were tested for solubility by separating the soluble and insoluble portions and electrophoresing them through a 10% SDS-PAGE gel stained with Coomassie Blue. Lane 1: Low molecular weight standard. Lane 2, XL1Blue cell control, un-induced. Lane 3, XL1Blue cell control, induced. Lane 4, pMalP vector, un-induced. Lane 5, pMalP, induced (whole extract). Lane 6, pMalP, induced (soluble fraction). Lane 7, pMalP, induced (insoluble fraction). Lane 8, pMalP/2FB Clone #1, un-induced. Lane 9, pMalP/2FB Clone #1, induced (whole extract). Lane 10, pMalP/2FB Clone #1, induced (soluble fraction). Lane 11, pMalP/2FB Clone #1, induced (insoluble fraction). Lane 12, pMalP/2FB Clone #2, uninduced. Lane 13, pMalP/2FB Clone #2, induced (whole extract). Lane 14, pMalP/2FB Clone #2, induced (soluble fraction). Lane 15, pMalP/2FB Clone #2, induced (insoluble fraction). Small arrow: Position of expressed MBP protein. Large arrow: Position of expressed and soluble MBP/2FB fusion recombinant protein.

Western blot analysis of the MBP/2FB fusion protein

To further characterize the soluble MBP/2FB fusion protein, Western immunoblot analysis was performed on the same samples analyzed by SDS-PAGE in figure 8e. The proteins were transferred onto immobilon membranes (in duplicate) and subjected to two different primary antibodies. In figure 9a, Respigam, a human high titered polyclonal anti-sera (obtained from MedImmune Inc.) was diluted 1:200 and absorbed to the immobilon. After developing, it was evident that there was a strong reaction with the proteins in the soluble and whole extract fractions (lanes 2 and 3), but not to the MBP cultures (lanes 5, 6 and 7). To ensure that this was not an unspecific phenomenon, a monoclonal antibody to MBP was used (obtained from the pMal expression kit, figure 9b), clearly reacting to the MBP/2FB fusion as well as the native MBP (with the fusion). Additional supporting data arose when the MBP monoclonal reaction in lane 5 increased in molecular weight from 40 KD to 53 KD when fused with the 2FB protein (lanes 2 and 3, figure 9b).

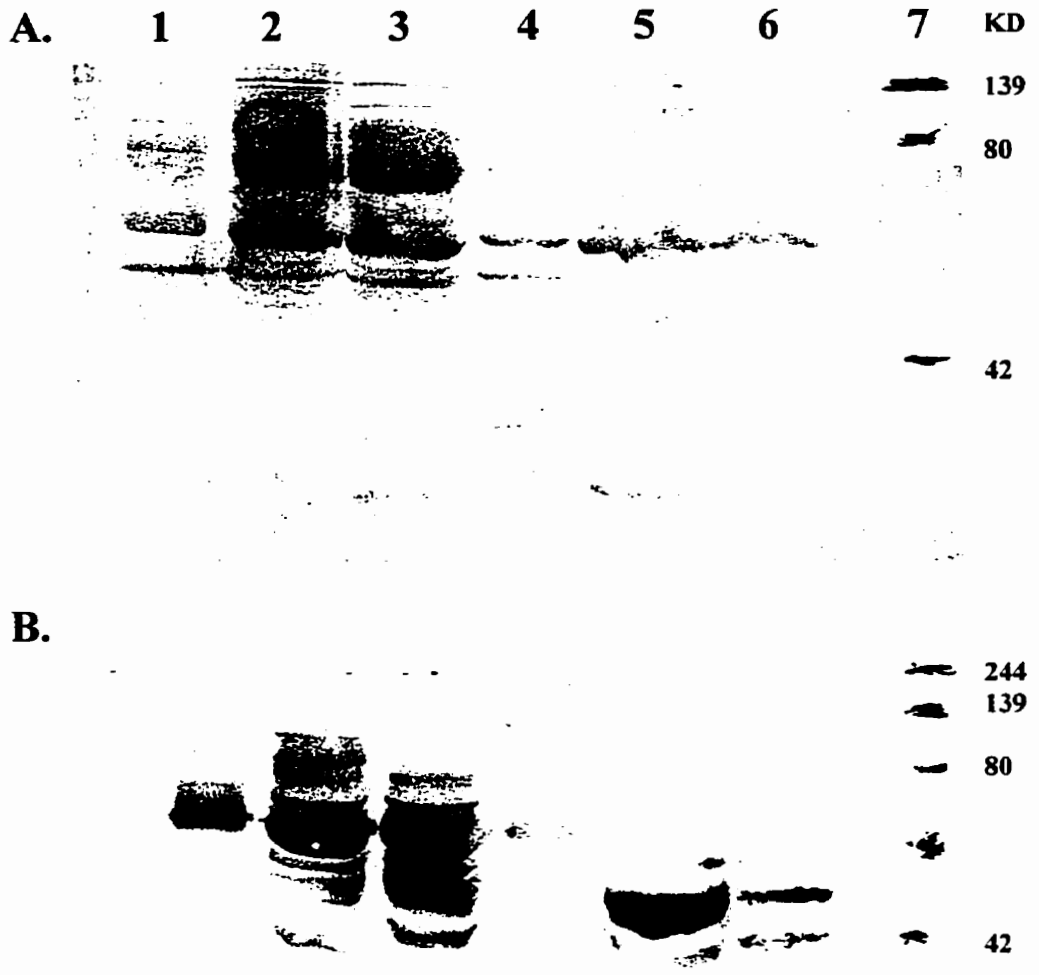

Figure 9. Assay of reactivity by Western Blot of MBP and MBP/2FB. Panels A and B represent MBP and MBP/2FB fusion protein cell extracts electrophoresed through a 10% SDS-PAGE gel, transferred to immobilon membrane and bound to either Respigam (panel A) or a monoclonal antibody specific to MBP (panel B). The lane numbers are marked as follows: Lane 1, pMalP/2FB Clone #1, induced (insouble fraction). Lane 2, pMalP/2FB Clone #1, induced (soluble fraction). Lane 3, pMalP/2FB Clone #1, induced (whole extract). Lane 4, pMalP/2FB Clone#1, uninduced. Lane 5, pMalP, induced (whole extract). Lane 6, pMalP, uninduced (whole extract). Lane 7, Biorad kaleidoscope pre-stained standards (marked in kilodaltons).

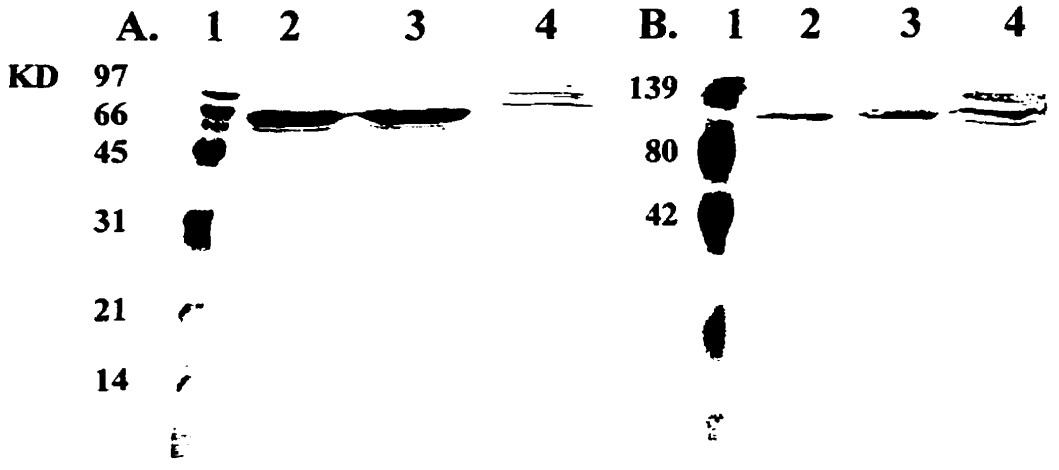

Figure 10. Purification of the MBP/2FB recombinant fusion protein. Panels A and B represent column purified recombinant protein electrophoresed through a 10% SDS-PAGE gel. Panel A was stained with Coomassie Blue for visualization. Panel B was transferred to Immoblin, and a Western Blot was performed using Respigam as the primary antibody, and developed using horse radish peroxidase.

Discussion

Analysis of pre-RSV season sera from children with cardiopulmonary disease

The purpose of analyzing sera from the children with underlying heart and lung disease was to look for a correlation between pre-season F ELISA antibody titers and subsequent RSV infection. For the subset of 73 infected and 73 uninfected children, the previously described observation was confirmed, in that neutralizing antibody titers greater than or equal to 1:100 were associated with a lower risk of subsequent RSV infection. Furthermore, this study extended these observations by showing that the F ELISA titer correlated significantly with the Lederle plaque reduction (PR) neutralization assay and that an F ELISA titer >1:10,000 was also significantly correlated with a decreased incidence of RSV infection. This argues strongly for the importance of antibody to the F protein in immunity to RSV.

The observation that antibody to RSV as measured by F ELISA was correlated with protection was similar to Siber et al's (25) observation in BALB/c mice as discussed in the introduction above (see page 10). The authors suggested that one explanation for the observed variability among assays in distinguishing protective from non-protective pools of human plasma was that different assays measured a distinct population of RSV antibodies. The microneutralization assay and the plaque reduction assay as used by Siber et al. differ mainly in the endpoint. The microneutralization assay measures the total quantity of RSV antigen associated with the cell layer by colorimetric development, as detected by ELISA (i.e. estimates the number of viral particles). In contrast, the plaque reduction assay counts the number of visible foci of viral induced syncytia. These foci do not necessarily correlate with total viral load or with pathogenicity. The ELISA F assay was almost as effective as the microneutralization

assay in identifying sera with animal protective activity. Yet the correlation between the two assays was weak albeit significant ($r=0.33$; $p<0.01$). A similarly poor degree of correlation was observed between the microneutralization and plaque reduction assays ($r=0.32$), whereas the F ELISA was not significantly correlated with the plaque reduction assay. In contrast to Siber et al.'s results, the F ELISA and neutralization assays from the current study strongly correlated ($r=0.72$-0.78, $p<0.001$).

There are several reasons why the PR assay results of the present study may differ from the study conducted by Siber et al. He used adult pooled sera which likely had broader polyclonal activity than sera from children aged less than 3 years. Thus the reactivity of the antibody population may have differed.

Although the F ELISA assay used by Siber et al. was similar to the one used here, the plaque reduction assay differed. The plaque reduction assay tested by Siber et al. used a methyl-cellulose overlay, crystal violet staining, and visual counting of plaques (foci). The Lederle assay used a sephadex overlay, tagged monoclonal antibodies and colorimetric identification of virus to visually count plaques. The endpoints of the two methods differ, such that the assay used by Siber et al. measures visible viral-induced syncytia, whereas the Lederele assay immunologically detects all virus present. Thus, the Lederle PR assay is close to the microneutralization assay and more equivalent in mimicking an in vivo situation.

Although the abilities of the microneutralization, and the Lederle PR assay to predict protection from RSV infection were similar, direct comparison is difficult since the correlates of protection were completely different. The former used animals directly challenged with RSV and the latter used human infants exposed in an unpredictable fashion to natural RSV in the community. However, the microneutralization assay was the basis for selecting pools of plasma

to make Respigam which has demonstrated protective efficacy in human trials. Furthermore, the F ELISA assay results were similarly protective for both systems.

Both neutralization and F ELISA assays measure antibody to RSV, but the F ELISA is much easier to perform and can be automated. Given the high correlation between the F ELISA and neutralization assay results and the association with protection from infection, the ELISA assay has potential for use as a screening test to identify high-risk children with high titers of antibody to F may who not require prophylaxis.

Analysis of acute sera from infants hospitalized with RSV infection

The purpose of analyzing acute sera from a population of RSV infected hospitalized infants was to determine significant correlations in a positive or negative direction between pre-existing antibody and RSV disease severity. Past studies have supported both a protective and disease enhancing role for antibody (2,36).

As shown in table 4, the majority of infected infants were younger than 13 weeks of age, and as such, any effect observed should be based on passively transferred maternal antibody. For the purposes of this study, significant disease severity was detected based on the incidence of early hypoxia. For both subgroup A and subgroup B infections, young age (<13 weeks) was significantly correlated with severe RSV disease. This finding is not surprising, in that it is consistent with both past and current RSV literature (37,41). For infants infected with subgroup B, antibody titer to the F protein as measured by ELISA assay was significantly inversely correlated with disease severity. Several F ELISA antibody titer breakpoints were examined by multivariate analysis, and infants with a titer greater than 1:1000 had a 5 fold less incidence of hypoxia, independent of age (table 5).

Glezen et al. (36) studied the risk of RSV infection for infants from low income families in relationship to age, sex, ethnic group, and maternal antibody levels. Results from that study demonstrated that severity of illness was inversely related to residual maternal neutralizing antibody. However, the association between high titers of F antibody, most likely maternal in origin, and reduced severity of infection is a new observation.

These results raise the question: Why are antibodies to an F protein derived from a subgroup A virus only protective among B infected infants? Failure to see a similar correlation for subgroup A infections could be related to the smaller number of subjects, that is the power to detect a difference among the 82 subgroup A infected infants is lower than among the 217 subgroup B infected infants. The proportion with hypoxia was similar among A and B infected infants (31-32%). However, the hypoxic infants with subgroup A infection were younger than those with subgroup B (81% versus 46% ≤12 weeks respectively) and the maternal antibody levels were higher. The dominant effect of young age could have masked a protective effect of antibody.

Another possibility is that the epitope reactivity for F proteins differ for subgroups A and B. Although the degree of homology between F proteins from A and B subgroups is quite high, there are sufficient differences to result in epitope variability. Arguing against this is the fact that the source of F protein used for the ELISA assay was an A subgroup RSV strain. This apparent paradoxical effect has no ready explanation but argues for further study of epitope reactivity involving both A and B derived F proteins.

Analysis of convalescent paired sera from previously healthy infants and children hospitalized with RSV infection

The paired sera were analyzed by the neutralization and F ELISA assays to assess the degree to which age and pre-existing antibody was correlated with convalescent homologous and/or heterologous antibody responses. By both functional and quantitative measures of antibody, younger infants (<6 months) had a blunted antibody response compared to older children (>6 months). This observation matches previously published data (1,2,4,36,37). In general, the homologous antibody response was better than the heterologous antibody response, arguing that epitopes may differ. Although the trend was more obvious in older infants, it was still seen in younger infants, despite a blunted antibody response.

The possible reasons why younger infants have poor homologous responses include:

(1) Immunological immaturity

(2) Suppression of immune responsiveness by maternal antibody

(3) Differences in immunity by infecting subgroup

(4) Severe disease may interfere with the immune response.

Given a large population of sera from patients with prospectively collected data on disease severity, the interaction between these factors could be analyzed by multiple logistic regression. This showed that disease severity was not a factor whereas age was key, and for B infected infants only pre-existing neutralizing antibody played a role.

The data concerning subgroup specific infections and passively transferred antibody are not consistent. For subgroup B infections only, the presence of homologous neutralizing antibody was associated with a blunted response. Murphy et al. (45), using a similar ELISA assay with F or G protein as the antigen demonstrated that age affects the response to the F protein whereas

the titer of pre-existing antibody affects the response to the G protein. The F proteins used in Murphy et al.'s study and the ELISA performed here were purified from the Long and A2 strains of RSV respectively, both of which are subgroup A strains. Although there is good homology between the two proteins, there are some differences which influence presentation of epitopes. The results of the current study extend Murphy et al.'s observation by establishing the presence of an age effect with respect to neutralization of whole virus.

Maternal antibody may interfere with the antibody response in several ways. There could be direct interference with immune processing, either by altering surrounding epitope sites, or by competitively inhibiting them. Neutralization of virus in vivo may reduce the antigenic stimuli for an immune response. As with observations made for antibody associated with hypoxia. significant correlations were found for subgroup B infected but not subgroup A infected infants. This could be due to the effect of smaller numbers or power to detect differences or to subgroup specific epitope differences.

Rationale for identifying and localizing F protein epitopes

In summary, the three studies presented above demonstrated that a high titer of antibody binding to the F protein is correlated with reduced disease severity in subgroup B infected infants, and a reduced risk of RSV infection. These observations clearly suggest that antibody to the F protein is important in immunity to RSV, but don't give further information about epitope reactivity in humans. To demonstrate epitope specific antibody responses, the next step was to examine differences in serum binding to distinct overlapping fragments of the F protein.

Unfortunately, after much effort, only the 2FB fragment could be expressed using the MBP fusion expression system. The observed reactivity with polyclonal Respigam implies there

are human epitopes within the fusion protein. Paradiso et al. (54) examined enzymatically cleaved fragments to identify epitopes on the F protein. An antibody binding site between amino acids 283-327 was identified that overlaps the junction between the 1FB and 2FB fragments. This may be the same site that reacted with Respigam. In order to confirm this data, the 2FB fragment should be cleaved from the MBP fusion partner site and re-tested by Western blot. Screening by a panel of monoclonal antibodies of known function and location would confirm that epitopes similar in structure to the native F protein exist on the 2FB fragment. Also, the 2FB fragment could be used as an antigen in an ELISA assay and tested in similar fashion as described above to determine whether any expressed epitopes are relevant for human disease.

The 2FB fragment was never of great interest in comparison to the 1FB and 3FB fragments due to the relative lack of epitope sites documented in the literature. Thus, expression of the first and third fragments was first attempted before comparison with the bank of sera was initiated. The attempt to express the first and third recombinant protein fragments could have been unsuccessful for several reasons. It is possible that expression of the fragments occurred, but they broke down before visualization on SDS-PAGE could be performed. The first fragment contains a signal peptide at the N-terminal end of the protein. This peptide, which is usually cleaved in its native form (14), could be toxic to many expression cell lines. This would inhibit cell line growth, and detection of the protein fragments would not occur. Despite these potential problems, expression of the entire first fragment was attempted since data on human epitope reactivity were so limited. It is questionable whether the signal peptide was solely responsible for the inability to express 1FB, because similar problems were encountered with 3FB.

Prokaryotic versus Eukaryotic expression systems

In choosing an expression system, both prokaryotic and eukaryotic systems were considered. Merits of the former included ease of manipulation, and the large quantities of expressed protein that could be obtained with amplification. There was at least one previously published report demonstrating success in expressing the RSV F protein in a prokaryotic system (reference to follow). The pET systems (Invitrogen Inc.) were tried first because they allowed native protein to be produced without a fusion partner. Subsequently, several fusion protein systems with reported success in the expression of toxic or unstable recombinant proteins, were tried. One of these included the Maltose Binding Protein fusion system. This system includes two plasmids that allow expression in the cell cytoplasm or the cell periplasm. Toxic or unstable proteins are better able to be expressed in the periplasm and this may have accounted for the success with 2FB.

A major obstacle common to all prokaryotic systems is the lack of carbohydrate processing necessary for some eukaryotic proteins. Eukaryotic systems were not initially attempted because they are expensive and time consuming. Given the lack of success in prokaryotic systems, a eukaryotic system was attempted.

The Baculovirus expression system combines the advantages of bacterial systems with the processing of eukaryotic ones. This insect cell system is capable of amplifying recombinant proteins in large amounts, and requires limited manipulation compared to traditional eukaryotic systems. The baculovirus expression system tried contains a histidine tag at the N-terminal portion of the recombinant protein which facilitates recovery of expressed product via metal chelation column purification. Although all recombinant F protein fragments were cloned into the helper vectors, DNA transfection into the insect cells was not successful, despite repeated

attempts. More effort needs to be directed towards this phase of research to see positive results.

Scrutiny of the cloning strategy

Given the repeated problems with cloning and expression of the three protein fragments, the entire cloning strategy was re-examined. In both prokaryotic and eukaryotic systems, the recombinant fragments would still be pieces of the whole native protein, and it is uncertain how closely they would mimic the native F protein. Each fragment represents a small portion of the whole protein. The folding and processing of a fragment versus the whole protein would most certainly be different, and could therefore affect epitope presentation.

There are two kinds of epitopes present on proteins, both of which are able to bind antibodies. Linear (continuous) epitopes are contiguous lengths of amino acids and are not affected by the final structure of the protein. In contrast, conformational (discontinuous) epitopes depend on folding, and final conformation of the protein for correct presentation (74). The study plan, involving expression of 3 separate overlapping peptides of the F protein, should have been adequate to examine linear epitopes, but could have posed problems for conformational epitopes depending on the processing and ultimate structure of the expressed fragments.

Other possible approaches

Although cloning and expression of the three F protein fragments were not successful, the results of the ELISA assays with the native F protein underscore the need for further examination of the epitopes recognized by human antibody. Other approaches could be used to determine which areas of the F protein are bound by human antibody. These include testing of nested peptides stabilized on polyethylene pins as used by Scopes et al. (53). Alternatively, sera could be

tested with digest products of the native F protein as done by Paradiso et al. (54). Both methods have potential problems associated with proper epitope presentation.

The recent demonstration that a humanized monoclonal antibody recognizing a single epitope site on the F protein is protective in humans (44) suggests another direction of research. This antibody could be used in competitive inhibition ELISA assays, to determine whether human sera contain antibody with similar patterns of reactivity. Specifically, using the same sera as described above, the following questions could be explored:

(1) Does the antibody cross the placenta?

(2) Do RSV infected infants make antibody as part of their convalescent response, and is that response age related.

(3) Does presence of the antibody confer protection against severe disease, as manifest by hypoxia?

(4) Is the antibody present, and does it correlate with protection in a group of children with underlying heart and lung disease?

Answers to these questions would not only further our understanding of epitopes relevant for human RSV disease, but also might point the way to an effective subunit vaccine.

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IMAGE EVALUATION
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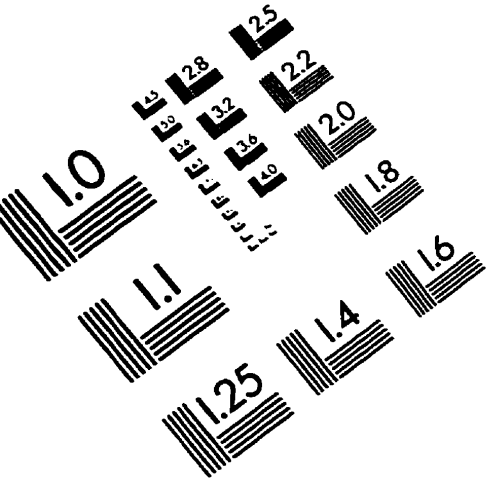

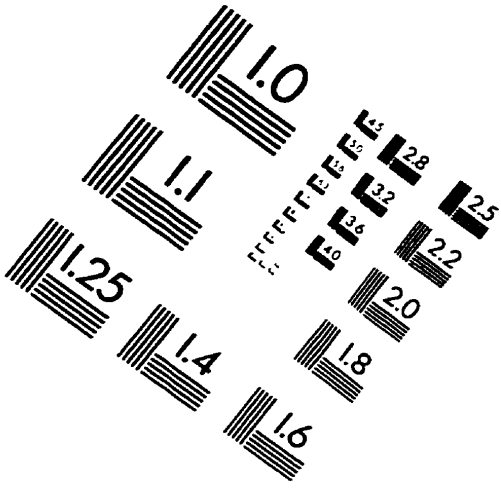

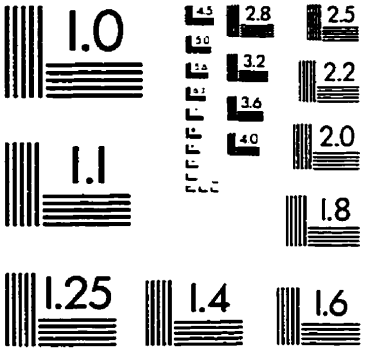

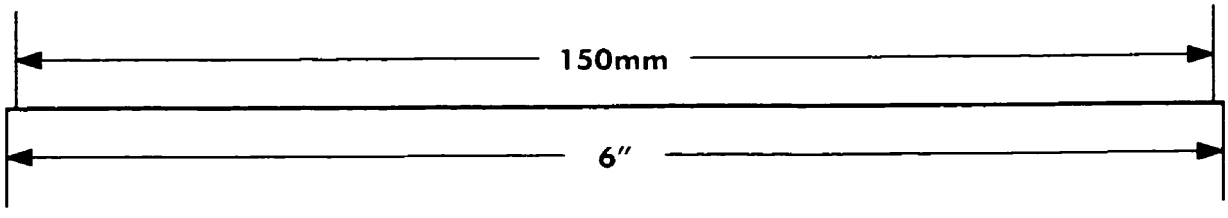

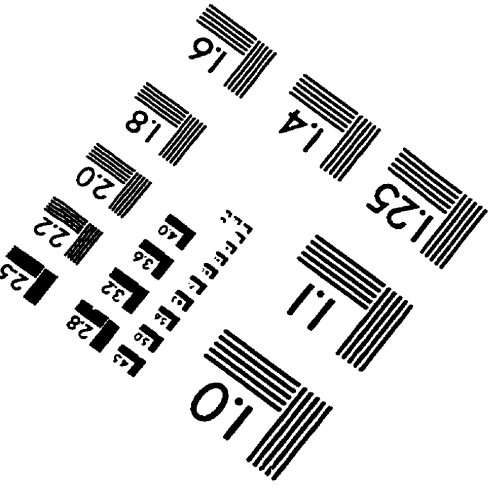

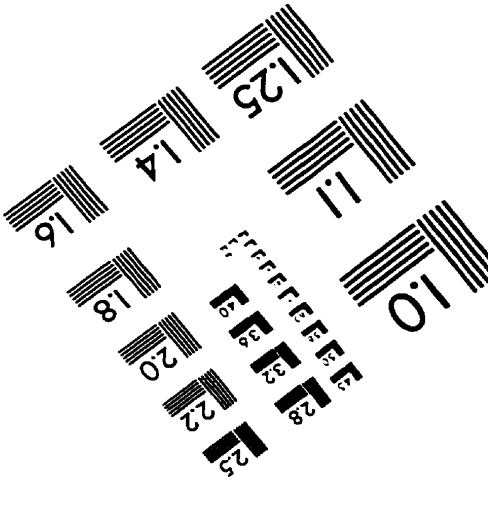

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