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Epitope specificities of human serum antibodies reactive with respiratory syncytial virus fusion protein

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Summary. Respiratory syncytial (RS) virus continues to cause serious human respiratory disease and no prophylactic vaccine is yet available. Serum antibodies to RS virus fusion protein (F) that have the appropriate specificities and activities could confer protection against severe RS virus infections. To explore human serum antibody responses to RS virus F we first characterised four epitopes on F and then measured the concentrations of human serum antibodies to these sites for 389 sera. Individuals varied in serum antibody concentration to the epitopes. The distribution patterns of the concentrations of antibodies reactive to each epitope were different. Antigenic variation of F at these epitopes in Southampton RS virus isolates was examined by immunofluorescence. The F proteins from different isolates varied within and between RS virus subtypes which co-circulated in the outbreak of winter 1985–1986. Variations in F detected by immunofluorescence were consistent with differences between the strains' susceptibilities to monoclonal antibody antiviral action.

Introduction

Respiratory syncytial (RS) virus, genus *Pneumovirus*, family *Paramyxoviridae*, is the major cause of severe lower respiratory tract infection (LRTI) in infants and young children [11]. Infection occurs worldwide, characteristically in regular yearly epidemics lasting 2–5 months. In urban areas of Britain 2.5% of babies required hospitalisation with RS virus infection [23]. Older children and adults are regularly reinfected but adults generally experience milder illness, although severe LRTI may occur in the elderly, chronically ill, or immunocompromised [10, 13].

RS virus has two major surface glycoproteins: a disulphide-linked, proteolytically processed, fusion protein F, M_r 70,000 [28], and an attachment protein G, M_r 90,000 [8]. Some monoclonal and polyclonal antibodies to these gly-

coproteins are able to neutralise RS virus infectivity in vitro and, on passive administration to animals, protect against infection [25]. Immunisation with purified F or G proteins or vaccinia virus expressing F or G proteins can also protect animals [18, 21]. The fusion protein has better prospects as a vaccine than G since it stimulates neutralising antibodies that are reactive with subtypes A and B of RS virus and because it stimulates better cytotoxic T cell responses [19].

Maternally acquired neutralising antibodies may account for the rarity of RS virus bronchiolitis and pneumonia in neonates. Thus babies with high levels of maternal antibodies experience primary infection that is delayed and of reduced severity [17] and women are prospective targets for vaccination. Caution is required with vaccine design since children vaccinated with formalin-inactivated RS virus, which induced non-neutralising antibodies had more severe illness on exposure to natural infection [7]. We found that volunteers with highest levels of neutralising antibodies and antibodies to particular epitopes on F were not susceptible to experimental RS virus infection [33], suggesting these antibodies protect.

The ability of RS virus to regularly re-infect susceptible adults may reflect virus strain variation, or may be because the specific response to infection is of very short duration, or dependent on HLA-linkage. To explore these possibilities, variation between individual immune responses to F, and also the antigenic variation of F were assessed. Initially four epitopes on F were characterised using monoclonal antibodies (MAbs); then concentrations of antibodies reactive to these four epitopes were measured in 389 sera by inhibition ELISA. This assay should allow quick assessment of serum antibody responses to other vaccines. The antigenic variation of F at these epitopes was determined in local clinical isolates using immunofluorescence.

Materials and methods

Viruses and cells

Edinburgh strain RS virus of subtype A [17] and 18537 virus of subtype B [4] and local isolates were grown in monolayer cultures of HEp-2 cells maintained in Dulbecco's minimal essential medium supplemented with 1% foetal calf serum (MM). Bulk growth of virus was performed as previously described [34].

Sera

Pre-natal sera were obtained from bloods sent to Southampton General Hospital for rubella status.

Monoclonal antibody production

Spleen cells from BALB/c mice immunised i.p. with 10^7 p.f.u. of Edinburgh RS virus infected HEp-2 cells were fused with BALB/c P3-NS-1-1Ag4-1 (NS-1) plasmacytoma cells with 50% (w/v) polyethylene glycol containing 5% dimethyl sulphoxide as described by Ward et al. [31]. Hybridomas producing F protein reactive antibodies were cloned by limiting

dilution and ascites fluid was produced by injecting phenotypically stable clone cells (10^7) i.p. into pristane primed BALB/c mice. MAbs were affinity purified from ascites fluid using protein-A-sepharose (Pharmacia) chromatography as described by Watanabe and co-workers [32].

Neutralisation assay

Sera and MAbs were treated at 56 °C for 30 min to inactivate complement; serially diluted four-fold in MM and mixed with 10^3 p.f.u. RS virus for 1 h at 37 °C. Fresh rabbit serum was added for complement assisted neutralisation. The mixture was adsorbed onto HEp-2 monolayers for 2 h, aspirated, and rinsed before overlaying with MM containing 0.75% (w/v) carboxymethyl cellulose (CMC). After 48 h the cells were stained with crystal violet and the syncytia counted by microscopy. Percentage syncytia reduction was determined for each serum dilution and the 60% neutralising dose (ND_{60}) calculated by interpolation [33].

Fusion inhibition assay

Monolayers of HEp-2 cells were stripped using 0.02% (w/v) EDTA in PBS, resuspended in MM, and infected with RS virus at m.o.i. of 10 in siliconised glass petri dishes. After 3 h adsorption the infected cells were harvested by gentle shaking, washed twice in 0.02% (w/v) EDTA in PBS and resuspended in MM. Uninfected HEp-2 cells were mixed with the infected cells at ratios of 25 : 1 and added to tissue culture trays. Ninety minutes was allowed for cell attachment, then the medium replaced with maintenance medium containing MAb and 0.75% (w/v) CMC. Syncytia were counted after 48 h.

Complement dependent cell lysis (CDCL)

^{51}Cr release cytotoxicity assays were performed to measure abilities of MAbs to cause CDCL of RS virus infected HEp-2. At 24 h post infection RS virus infected HEp-2 cells were washed and mixed with [^{51}Cr]sodium chromate (Amersham International plc) at 1.75×10^7 cells per mCi for 1 h at 37 °C. After extensive washing cells were aliquoted into 96 v-well trays at 10^3 c.p.s. per well. Serial $\sqrt{10}$ dilutions of antibodies (0.03 to 100 µg per ml in MM) were mixed with the cells and complement for 1 h at 37 °C. The cells were then pelleted 1000 **g** for 1 min in a GF8 Centrifuge and the released ^{51}Cr counted. Results were expressed as a percentage of the counts released by 10% (w/v) saponin detergent under identical conditions. Percentage ^{51}Cr release for each antibody dilution was plotted against $\log_{10}$ antibody concentration and the concentration causing 50% maximum release was determined by extrapolation.

Immunofluorescence

RS virus infected cells or mock infected cells were washed three times in PBS by centrifugation at 500 **g** for 10 min. 10 µl cell suspension in PBS (5×10^3 cells) was added to multispot microscope slides (C. A. Hendley (Essex) Ltd.); air dried; acetone-fixed; and stored at −70 °C. After rinsing with PBS, bovine anti-RS virus serum (Wellcome Reagents) diluted 1 : 10 in PBS or MAb diluted 1 : 100 in PBS were added to wells for 30 min (20 µl/well) at 37 °C. Following washing, fluorescein isothiocyanate conjugated (FITC) antisera [FITC-sheep antiserum to bovine IgG (Wellcome Reagents); or FITC-rabbit antiserum to mouse IgG (Dako immunoglobulins a/s)] were incubated for 30 min at 37 °C diluted 1 : 40 in PBS containing 0.1% (w/v) naphthalene black (20 µl/well). After washing, glass coverslips were applied, and the wells examined with an Ortholux II Fluorescent Microscope (E. Leitz, Wetzlar, Federal Republic of Germany) equipped with a HBO-200 mercury vapour lamp and Ploem 1 incident illumination.

Enzyme linked immunosorbent assay (ELISA)

ELISAs were performed in high-activated PVC immunoassay plates (ICN Flow Ltd.) at 37 °C using 100 μl volumes throughout. Fusion protein was cholate-extracted from RS virus infected HEp-2 cells and immunoaffinity purified [34]. Antigen was diluted in coupling buffer (100 mM $Na_2CO_3/NaHCO_3$ plus 0.05% w/v sodium azide, pH 9.6) and coupled to wells by incubation for 16 h at 20 °C. Extensive washing was performed between each stage with PBS-W1 [PBS + 0.05% (w/v) polyoxyethylene ether-W1]. Primary antibodies and HRPO-linked antisera were reacted in PBS-W1 containing 5% (v/v) rabbit serum (ELISA-diluent). TMB substrate (0.1 M sodium acetate pH 6.0 + 0.15 mM 3,3′,5,5′-tetramethyl benzidine + 0.005% H_2O_2) was incubated for 5 min, then 50 μl 2M H_2SO_4 added to stop reaction. ELISA A_{450} was measured by Titretek Twinreader (Flow Laboratories) and converted to antibody concentration (μg/ml) using standard curves constructed as described previously [34].

F protein epitope mapping

HPLC-purified MAbs (1 mg/ml in 150 mM phosphate buffer pH 7.0) were biotinylated with biotin-n-hydroxysuccinimide ester (BnHS; BRL, Maryland, U.S.A.) freshly dissolved in dry dimethylformamide to give a BnHS to protein mass ratio of 1:5. The reaction was stopped after 23 min at 20 °C by adding 1 M NH_4Cl to a final concentration of 0.114 mM. Biotinylated antibodies at 90% maximum binding capacity were mixed with an equal volume of unlabelled MAb at ten times this concentration and transferred to fusion protein-coated wells and incubated at 37 °C for 1 h. After four washes the amount of biotinylated antibody bound to F in the presence of competing monoclonal was detected using streptavidin-biotinylated-HRPO complex (Amersham International, plc). Percentage inhibition by competing antibody was determined from the difference between the mean A_{450} obtained with (i) and without (u) competition, as a percentage of homologous inhibition (h):

$$\text{percentage inhibition} = 100 \times (A_{450}u - A_{450}i)/(A_{450}u - A_{450}h).$$

Inhibition assay to measure serum antibodies reactive to specific F protein epitopes

Fusion protein was attached to wells by incubation for 16 h at 20 °C in coupling buffer (1 μg/ml). Serially diluted sera were reacted for 2 h, the well rinsed with PBS, and MAbs(F1, F2, F3 or F4) reacted at 10 μg/ml in ELISA-diluent for 1 h. The bound MAbs were detected using murine specific HRPO-linked anti-mouse IgG $F(ab')_2$ (Amersham International plc). Twelve control wells lacking serum were included on each tray.

The mass of serum antibodies reactive with F1, F2, F3, and F4 epitopes was determined as the difference in mass of MAbs bound with and without serum. The serum dilution nearest to allowing 25 ng MAb to bind to F ($A_{450} = 0.5$) was used to calculate final serum concentration. Masses of antibodies bound were determined from A_{45} using standard curves.

Results

Fusion protein epitope characterisation

Epitope mapping

MAbs F1, F2, F3, and F4 which specifically immunoprecipitated F from RS virus-infected HEp-2 cells, and gave different inhibition patterns with a panel of ^{125}I-labelled antibodies (data not shown), were biotinylated for epitope mapping. The percentage inhibitions of biotinylated antibodies binding to F by unlabelled MAbs (see Fig. 1) confirmed their epitopes are distinct.

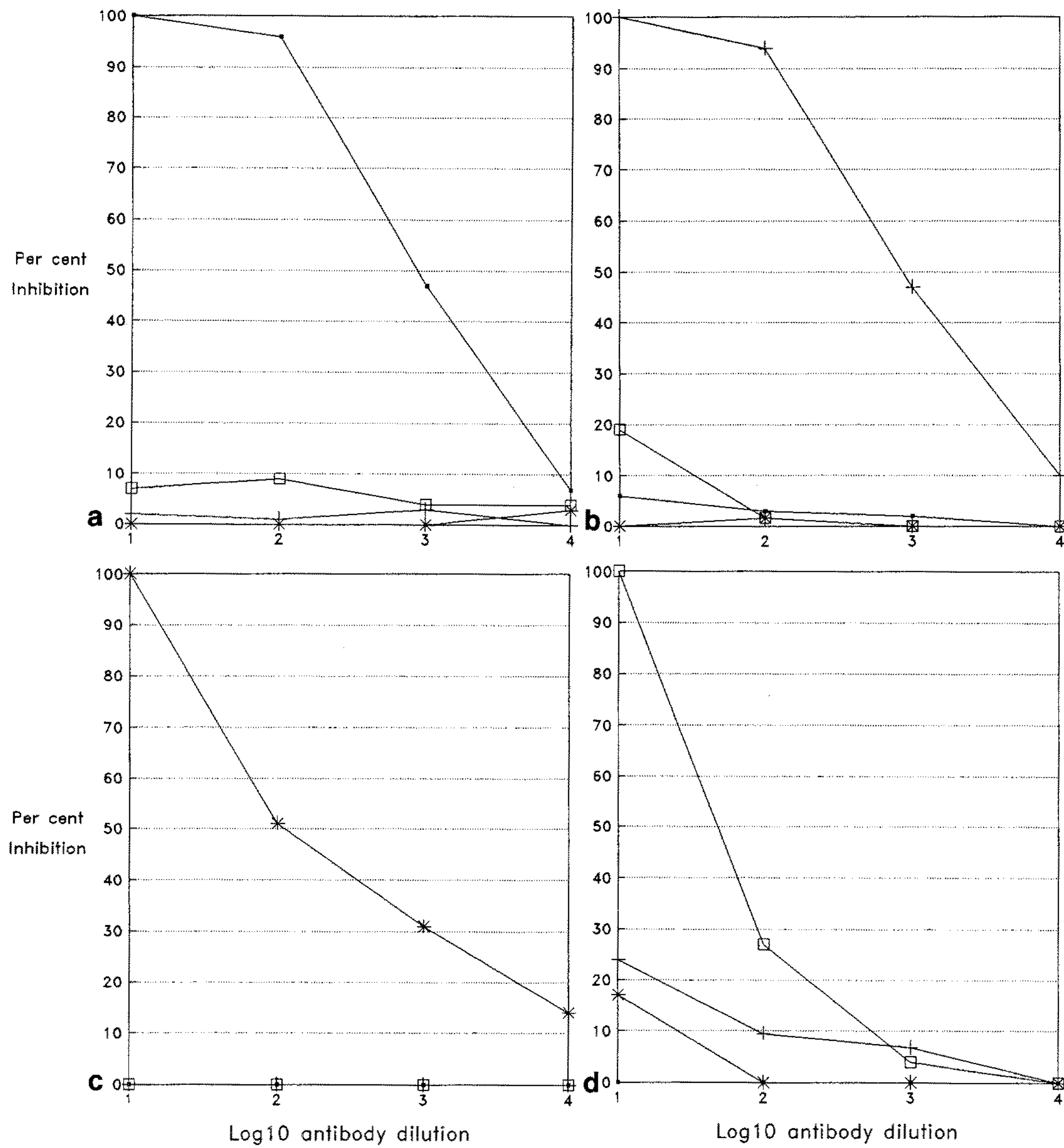

Fig. 1. Competitive inhibition between F1, F2, F3, and F4 monoclonal antibodies. Inhibition of **a** F1, **b** F2, **c** F3, and **d** F4. Biotinylated MAbs were mixed with serial ten-fold dilutions of unlabelled MAbs and transferred to fusion protein coated ELISA wells. The amount of biotinylated antibody bound was detected using streptavidin-biotin-peroxidase complex in ELISA. Percentage inhibitions are plotted against $\log_{10}$ dilution of competing antibody: ■ inhibition by unlabelled F1; + inhibition by unlabelled F2; * inhibition by unlabelled F3; □ inhibition by unlabelled F4

In vitro antiviral properties of the MAbs

In vitro antiviral assays were performed with MAbs to measure their neutralising (both with and without complement), fusion inhibiting, and CDCL activities

against laboratory strains and clinical isolates of both RS virus subtypes (see Table 1).

The MAbs were chosen to be of appropriate antibody subclass to bind complement (F1 was subclass IgG_{2b}; F2, F3, and F4 were subclass IgG_{2a}). MAbs F3 and F4 neutralised all the virus strains in the absence of added complement whereas MAbs F1 and F2 were unable to neutralise RS virus strains without complement. MAbs F3 and F4 possessed fusion inhibition activity whereas MAbs F1 and F2 failed to inhibit fusion. Subtype B viruses were more resistant to the fusion-inhibition activities of these MAbs than the subtype A strains. All MAbs caused total complement enhanced neutralisation

Table 1. In vitro anti-viral properties of the monoclonal antibodies

	Assay	Monoclonal antibody			
		F1	F2	F3	F4
Subtype A virus					
Edinburgh	N	0	0	100	100
	N + C	98	99	100	99
	F	0	0	95	94
	CDCL	0.20	0.45	0.65	1.00
3650	N	0	16	100	100
	N + C	100	100	100	100
	F	0	0	100	96
	CDCL	0.30	0.45	0.90	0.30
Subtype B virus					
8/60	N	0	0	100	98
	N + C	68	95	100	95
	F	0	0	65	79
	CDCL	<0.10	<0.10	1.00	1.00
3093	N	7	13	100	100
	N + C	100	100	100	100
	F	0	0	25	40
	CDCL	0.40	<0.10	0.75	1.00

For neutralisation assays (N), 1 ml MAb (10 µg/ml) was mixed with 10^3 p.f.u. RS virus for 1 h at 37 °C and added to HEp-2 cell monolayers. Percentage plaque reductions compared to control wells after 48 h are presented. Complement assisted neutralisation (N + C), was performed similarly except 0.15 ml fresh rabbit serum was included in each well. For fusion inhibition assay (F), RS virus infected HEp-2 cells were mixed with normal HEp-2 cells (ratio 1:25) and overlayed with 1 ml 1.3% CMC in MM containing MAb (1 µg/ml). Percentage reduction in the number of syncytia compared to controls after 48 h are presented. Complement dependent cell lysis (CDCL) was measured by reacting $\sqrt{10}$-fold dilutions of antibodies (100 µg/ml to 0.10 µg/ml) with ^{51}Cr-labelled HEp-2 cells infected with each virus strain. The mass of antibody (ng) causing 50% maximum release (obtained by plotting ^{51}Cr release against $\log_{10}$ antibody mass) are presented

of the virus strains tested apart from F1 and F2 MAbs which were less efficient against the 8/60 strain (68% and 95% neutralisation respectively). All four MAbs were effective at complement mediated cell lysis of HEp-2 cells infected with each of the virus strains tested. F3 and F4 were less active at CDCL than F1 and F2 particularly against cells infected with subtype B RS virus. The differences between isolates in their susceptibility to antibody antiviral action suggests that antigenic variation of F protein may reduce antibody binding and therefore activity.

Concentrations of human serum antibodies reactive to F protein epitopes

The competitive inhibition assay measures the concentration of MAb blocked from binding to F by human serum antibodies. In addition to serum antibodies which bind to the epitope itself, the assay may, to a lesser extent, detect antibodies which bind to overlapping epitopes, and also other antibodies which interfere with MAb binding. The serum antibody concentrations quoted in this paper therefore give a measure of the levels of antibodies directed to the sites, rather than being the absolute concentrations of epitope specific antibodies.

The competitive inhibition ELISAs demonstrated great variation between the concentration of antibodies to the different F epitopes for the 389 pre-natal sera. All of the sera contained antibodies reactive to F3 epitope, whereas twelve sera lacked antibodies reactive to the F2 epitope, thirteen lacked antibodies reactive to F4 epitope, and twenty-five sera (6.4%) lacked detectable antibodies reactive to F1 epitope. The results for eight sera, selected to show that sera highly reactive with one or more epitopes may be poorly reactive to other epitopes on the same antigen, are given in Table 2.

The distributions of serum antibody concentrations for this population of pregnant women was determined by plotting the number of individuals with epitope specific antibody concentrations within sequential 3 μg/ml concentration ranges (Fig. 2). Both the F1 and F2 reactive antibody concentrations within the population produced skewed Gaussian distributions. The similarity of F1 and F2 distributions was reflected by their comparative values for the population range, mean and standard deviations (F1 epitope: range = 55.2 μg/ml, $\bar{x}$ = 10.80 μg/ml, SD = 8.96 μg/ml; F2 epitope: range = 67.7 μg/ml, $\bar{x}$ = 12.24 μg/ml, SD = 9.08 μg/ml). 17% of the population had antibody levels to F1 epitope below 3 μg/ml and 13% of the population had antibodies reactive to F2 epitope below this value. The distributions for concentrations of antibodies reactive with F3 and F4 epitopes were both narrower in range than the F1 and F2 distribution. The majority of the population possessed relatively low concentrations of antibodies reactive to F4 (range = 25.10 μg/ml, $\bar{x}$ = 5.10 μg/ml, SD = 4.54 μg/ml) and 40% had less than 3 μg/ml. In contrast the F3 epitope reactive antibodies had a Gaussian distribution pattern with a higher population mean value (range = 39.20 μg/ml, $\bar{x}$ = 12.78, SD = 4.46) with only 2% having less than 3 μg/ml.

Correlation coefficients (c) between the serum antibody concentrations re-

Table 2. Antibody concentrations (μg/ml) reactive to different epitopes on RS virus fusion protein in selected sera

Patient	Serum antibodies reactive with epitope			
	F1	F2	F3	F4
A	0.00	0.00	15.30	11.55
B	0.00	13.80	19.50	1.05
C	0.00	36.30	14.25	19.95
D	15.75	0.00	15.30	12.60
E	13.35	13.05	0.90	0.00
F	21.60	1.35	10.50	0.00
G	26.55	32.70	19.50	15.45
H	44.70	29.55	16.95	16.50

The concentrations of serum antibodies reactive to the epitopes on fusion protein defined by the monoclonal antibodies F1, F2, F3, and F4, were determined in 389 prenatal sera by competitive inhibition ELISA. The concentration of serum antibodies reactive to a particular epitope was calculated as the concentration of F1, F2, F3, or F4 displaced from binding to F by the serum. The values for eight selected sera (μg/ml) are presented to demonstrate that individuals varied in their serum antibody concentrations to the different fusion protein epitopes

active to the four epitopes were different in value and highly significant. The highest mean correlation coefficient was for antibody concentrations reactive to F2 and F4 epitopes (c = 0.655) and the monoclonal antibodies reactive to these competitively inhibited each other by 22%. The lowest correlation (c = 0.162) occurred between antibody concentrations to F1 and F3 epitopes defined by monoclonal antibodies which did not competitively inhibit binding to fusion protein (F1, F2 c = 0.492; F1, F4 c = 0.439; F2, F3 c = 0.334; F3, F4 c = 0.238). These correlation coefficients confirm marked individual differences in the epitope specificity of the immunodominant antibody response to F protein antigen in natural infection.

Antigenic variation of F

The antigenic variation of F from Southampton clinical isolates collected during the outbreak of winter 1985–1986 was assessed by immunofluorescence. Virus isolates were grown in HEp-2 cells; acetone-fixed to slides and characterised by indirect immunofluorescence with a panel of MAbs (see Table 3). The immunofluorescence tests were read under code and confirmed by an independent observer. A2 (subtype A) and 8/60 (subtype B) RS viruses were included as positive controls and uninfected cells as negative controls. Cells were confirmed to be well-infected with RS virus strains by immunofluorescence using bovine anti-RS virus serum (Wellcome) before assaying with MAbs. Thus differences detected by IF could not be due to variation in virus growth. Results were

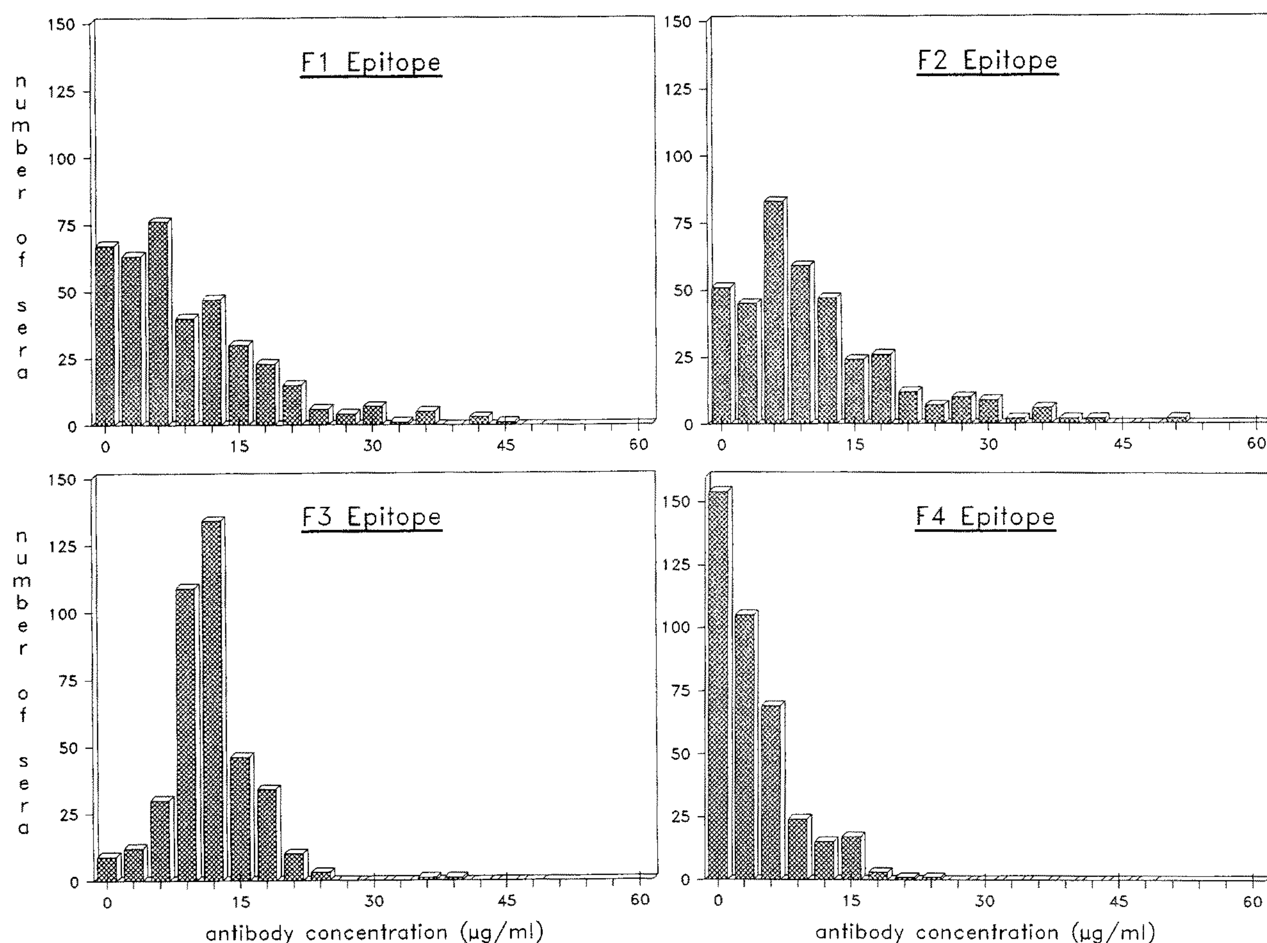

Fig. 2. Distribution of serum antibody concentrations reactive with different epitopes on RS virus fusion protein for a population of 389 women. Serum antibody concentrations reactive with F1, F2, F3, and F4 epitopes on fusion protein were measured in 389 prenatal sera by competitive inhibition ELISA. The population distribution patterns of serum antibody concentrations to each epitope are plotted to show the number of individuals having epitope specific serum antibody concentrations within sequential 3 µg per ml IgG concentrations

scored as negative (−) or graded positive (+ to + + + +) and are reported as either negative or positive for clarity (Table 3). Of nineteen local RS virus isolates tested, five (26%) produced membrane fluorescence with subtype B specific MAbs (i.e. were subtype B). The subtype B isolates gave positive membrane fluorescence with F1 and F4, and two reacted with F3. None of the subtype B viruses reacted with F2 suggesting that changes in this epitope are sufficient to abolish antibody binding in immunofluorescence assay.

Fourteen of the nineteen local isolates tested gave subtype A reaction pattern. Ten of these subtype A viruses reacted with all antibodies, while four isolates produced a different reaction pattern (see Table 3). Thus, the fusion proteins

Table 3. Reactivity of monoclonal antibodies with local RS virus isolates by immunofluorescence

Virus	Isolation date	Reactivity by immunofluorescence							
		F1	F2	F3	F4	F(A)		F(B)	
						4064	3784	3179	3206
Subtype A									
A2	prototype	+	+	+	+	+	+	−	−
36424	01:11:85	+	+	+	+	+	+	−	−
39123	22:11:85	−	−	−	+	+	+	−	−
39124	22:11:85	+	+	+	+	+	+	−	−
40078	02:12:85	+	+	+	+	+	+	−	−
41637	12:12:85	+	+	+	+	+	+	−	−
41700	12:12:85	+	+	+	+	+	+	−	−
41897	16:12:85	+	+	+	+	+	+	−	−
42142	17:12:85	+	−	+	+	+	+	−	−
42816	20:12:85	+	+	+	+	+	+	−	−
42983	24:12:85	+	+	+	−	+	+	−	−
46572	28:12:85	+	+	+	+	+	+	−	−
1093	10:01:86	+	+	+	+	+	+	−	−
1126	13:01:86	−	+	−	+	−	+	−	−
1375	14:01:86	+	+	+	+	+	+	−	−
Subtype B									
8/60	prototype	+	−	−	+	+	+	+	+
39245	25:11:85	+	−	−	+	+	+	+	+
42900	23:12:85	+	−	−	+	+	+	+	+
26	02:01:86	+	−	+	+	+	+	+	+
909	09:01:86	+	−	−	+	+	+	+	+
1202	13:01:86	+	−	+	+	+	+	+	+

HEp-2 cells infected with RS virus isolated in Southampton during the winter of 1985–1986 were acetone-fixed to slides and assayed by indirect immunofluorescence using F specific MAbs. MAbs specific for F subtype A virus (4064 and 3784) and for subtype B virus (3179 and 3026) were kindly provided by Dr. B. F. Fernie, Dept. Microbiology, Georgetown Universtiy, Maryland, U.S.A.

+ Positive membrane immunofluorescence, − no reaction

from RS viruses isolated in Southampton during a single epidemic varied within the subtype A and B groupings. The variation in F was an important finding since this protein has previously been considered relatively stable to antigenic change [15, 29]. Isolates of both A and B subtypes were found to co-circulate throughout the entire outbreak.

The F4 epitope was most highly conserved with only one of nineteen isolates failing to react with F4 MAb; F1 reacted with all but two virus isolates; while F3 failed to react with six (32%) of the strains. F2 reacted with all subtype A isolates but none of subtype B viruses by immunofluorescence.

The different reaction patterns of monoclonal antibodies by immunofluorescence is most likely caused by antigenic variation of F. F3 MAb caused only partial fusion inhibition of 8/60 in cell culture and failed to react with this strain by immunofluorecence. This suggests that antigenic variation of F in 8/60 reduced the binding of F3. Further denaturation of F in IF probably by acetone fixation was sufficient to prevent reaction altogether.

Discussion

Antigenic sites on RS virus fusion protein have been characterised. MAbs to F with fusion inhibiting and virus neutralising activities, and MAbs with only complement-dependent neutralising activity have been described. Our fusion inhibiting and neutralising MAb (F3) reacts with F_1 subunit (M_r 48,000) and a M_r 20,000 protein on reduced Western blots (not shown) probably binds to same antigenic region as fusion inhibiting monoclonals described by other groups which behave similarly [3, 9, 27, 29]. These MAbs are highly protective against RS virus infection in animals [26].

This is the first study of the immunogenicity of different fusion protein epitopes in humans. The results confirm that the four epitopes defined by murine MAbs are immunogenic in humans. Individuals varied in their serum antibody concentrations reactive to the different epitopes, but most people possessed antibodies reactive with all four epitopes, and none of the epitopes appeared immunodominant. The degree of variation between individuals is surprising considering people inevitably encounter multiple infections with RS virus. The antigenic variation of F (as we demonstrate here by immunofluorescence) may account for differences in antibody levels to the different epitopes, although differences in individual responsiveness may also be important. Antibody responses to influenza virus haemagglutinin also vary between individuals [30]; one study showed 41% of adult and 58% child sera failed to recognise viruses with HAs having single amino acid substitutions [16].

RS virus antigenic variation differs from influenza virus variation because there are no major antigenic shifts to produce pandemic strains. The two subtypes of RS virus have evolved separately for a long time [15]. However the indirect immunofluorescence did reveal antigenic variation in F and demonstrated heterogeneity of viruses within an outbreak. Immunological selective pressures causing antigenic variation of F, and the induction of different immune responses by the variant viruses may occur together.

The analysis of antigenic variation by immunofluorescence revealed a high degree of variation in the fusion protein of strains isolated in Southampton. Of fifteen subtype A viruses two viruses failed to react with each of F1, F2, and F3 epitopes (six strains altogether), and one failed to react with F4. For the subtype B isolates, all six reacted with F1 and F4, none reacted with F2 and four failed to react with F3. The variation detected by immunofluorescence appeared to correlate with susceptibility of isolates to fusion inhibition by antibodies. Thus 8/60 which failed to react with F3 in IF was more resistant

to fusion inhibition by this antibody. Sequencing of escape mutants to other antibodies suggest minor, possibly single, amino acid changes not necessarily within the epitope, may account for these differences [9].

It is important to know whether minor antigenic changes in F have clinical or immunological significance in human infections; antigenic variation of F may allow RS virus to re-infect adults. Antibodies produced following experimental infections with RS virus reacted equally well in ELISA with fusion protein of subtype A and subtype B [33]. Other work indicated that neutralising reponses to F are subtype cross-reactive [6, 20]; and CTL responses to F also appear to be cross-reactive [2]. Some reports have suggested natural immunity in humans protects more against the most recently infecting subtype [14]. Larger studies are required to confirm both this and also reports suggesting that subtype A viruses are more virulent and infect younger infants [22, 24].

A critical question for RS virus vaccine development is whether poor responses to protective epitopes on RS virus infection protein are in part genetically determined. For example, a lack of response to hepatitis B vaccine was associated with homozygosity for the HLA-B8, DR3 haplotype suggesting this caused HBsAg to fail to bind to DR3 associated class II [1]. The levels of antibodies in pre-natal sera suggest there is no such population of "low responders" to these fusion protein epitopes. The fact that all sera contained antibodies to at least one epitope (F3) suggests that all humans recognise the T_H epitopes on F. The results do not exclude the possibility that some people fail to respond to certain B cell epitopes on F.

Further analysis of sera from volunteers in the RS virus *ts* mutant vaccine trial [12, 33] suggests that critical levels of serum antibodies reactive to F1 and F3 epitopes provide protection against infection (B. S. Robinson, unpubl. data). Volunteers with levels of serum antibodies to F1 $> 8.0\,\mu g/ml$ and antibodies to F3 $> 8.5\,\mu g/ml$ appeared to be protected from experimental RS virus infection and clinical symptoms. 203 (59%) and 339 (87%) of the pre-natal sera assayed in this study contained these or higher levels of antibodies to F1 and F3 epitopes respectively. It is of interest that these percentages are the same as the proportion of adult contacts spared from respiratory tract infection [5].

The relatively high values and narrow ranges of antibody concentrations to the four epitopes in pre-natal sera, as well as the small number of individuals lacking these antibodies (particularly F3) suggest people could be readily boosted to protective levels by appropriate vaccination. This hopefully would provide the neonate with sufficient maternal protective antibody to prevent life-threatening infection until he/she may be immunised. Prospective vaccines will require careful assessment to see they induce appropriate antibody responses.

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