Synthetic peptides corresponding to the F protein of RSV stimulate murine B and T cells but fail to confer protection

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Summary. We have previously located a major neutralization site of the fusion protein of respiratory syncytial virus (RSV) in the polypeptide region extending from amino acids Ile221 to Glu232. In this report, 8 peptides corresponding to the six major hydrophilic regions of the F_1 subunit were selected to analyse their immunogenic and protective capacities as well as their ability to block the high neutralization activities of 4 monoclonal antibodies (MAbs). Only 5 of the 8 peptides tested induced specific antibodies while all induced an in vitro interleukin-2 response of splenocytes from immunized mice. Peptide 3 (Ile221-Phe237) was able to elicit neutralizing antibodies, confirming our previous hypothesis concerning the location of a neutralization site. However, immunization with the latter did not induce significant reduction of virus in lungs of BALB/c mice upon challenge, probably due to an inadequate level of circulating neutralizing antibodies. Interestingly, peptides 2 (Asn216-Glu232), 3 (Ile221-Phe237), and 5 (Ser275-Ile288) blocked in vitro neutralization by four different F_1 specific MAbs. A hypothesis is proposed to explain these results.

Introduction

Respiratory syncytial virus (RSV) is an important human and bovine pathogen of the respiratory tract. The virus is classified in the family *Paramyxoviridae*, genus *Pneumovirus* [17]. Previous antigenic studies with monoclonal antibodies (MAbs) have suggested a predominant role for the fusion glycoprotein F in inducing protection [1, 4, 13, 14, 16, 18, 20, 23–25]. This hypothesis was confirmed in experiments involving the expression of the fusion protein F in

recombinant vaccinia virus as compared with the expression of the major gly-coprotein G [12].

Örvell et al. [13] have reported that most of the neutralizing antibodies among 70 different MAbs were effective in protecting against the human A and B and bovine strains of RSV and also reacted in immunofluorescence tests with the related caprine strain [6]. Other workers have found that neutralization was not the only mechanism of protection [18] and that not all the neutralizing antibodies were protective in vivo [26]. Similarly, we have shown that ISCOM subunit vaccines comprising the fusion protein of either human or bovine RSV could efficiently induce neutralizing antibodies that were equally active against both isolates [22].

The importance of the F protein in protection has encouraged studies to characterize the specific regions involved in protection, particularly since they were presumably conserved among isolates. We have used our efficient neu-tralizing MAb 7C2 to identify a major neutralization epitope of the F protein that was conserved in the human, bovine and caprine RSV isolates [20, 23]. The epitope, or part of it, was localized in the Ile221-Glu232 region of the F_1 subunit of the fusion protein. Lopez et al. [8] have also determined that two Asn residues located at positions 262 and 268 were important in conserving the integrity of a major epitope that reacts with their highly neutralizing MAb-47F. Finally, Scopes et al. [15] have identified a linear epitope in the region comprising Phe483 to Phe488 of the F protein that reacts with convalescent serum.

To pursue these studies we have analysed the immunogenic and protective capacities of 8 synthetic peptides selected on the basis of the hydropathicity profile of the fusion protein of RSV. Only five of 8 peptides tested induced specific antibodies as well as in vitro interleukin-2 responses in splenocytes isolated from RSV immunized mice. Furthermore, the peptide corresponding to the Ile221-Phe237 region elicited neutralizing antibodies although it was inefficient in conferring protection. Also, three peptides were shown to react to varying degrees with four related and highly neutralizing MAbs directed against the F_1 subunit of the RSV fusion protein.

Materials and Methods

Cells and virus

The Long (ATCC VR-26; American Type Culture Collection, Rockville, Md., U.S.A.), the bovine strain A-51908 (ATCC VR-794) and A2 human strains of RSV were propagated in HEp-2 cells (ATCC CCL-23) previously grown in equal parts of Eagle's minimal essential medium and of medium 199, supplemented with 50 µg/ml of gentamicin and 5% fetal calf serum.

Monoclonal antibodies

BALB/c mice (Charles River, St Constant, Québec, Canada) were either immunized by three successive instillations (50 µl) of either Long or A2 human or Snook bovine RSV

suspensions (10^6 pfu/ml) and/or by injection either in the foot pad or intraperitoneally of 2×10^5 BALB/c cells persistently infected with the Long strain virus, administered in Freund's incomplete adjuvant or saponin. Splenocytes were fused with NS-1 myeloma cells in the presence of polyethylene glycol [27] and the resulting hybridomas were selected either by ELISA, RIA or neutralization assays. Ascitic fluids were produced by injection of pristane pre-treated BALB/c mice with 10^6 cloned cells. MAbB4 is a monoclonal antibody to bovine RSV [5] and MAb11 has been described previously [18].

Competitive ELISA assays

Neutralizing MAbs (ascitic fluid) that were specific for the fusion protein of human RSV were used in competitive binding enzyme-linked immunosorbent assays. Briefly, various amounts of unlabeled competing MAbs (1 to 500 ng total IgG) were added in microtiter plates previously coated with 200 ng of RSV purified by sucrose density step gradient centrifugation (30/50%). After incubation during 2 h at 20 °C, the plates were washed and 100 µl of horseradish peroxidase-labeled MAb 7C2 added to each well in the same quantity. The plates were incubated 2 h at 20 °C, washed again and the reaction developed with O-phenylenediamine (0.4 mg/ml of 0.1 M sodium citrate pH 5 containing 0.03% H_2O_2).

Passive protection studies

Groups of five BALB/c mice, six to eight weeks old, were injected intraperitoneally with 0.1 ml of ascitic fluid. The next day they were challenged intranasally with $10^{4.2}$ pfu of the A2 strain of RSV. Five days later the lungs were assayed for the presence of virus [18, 19].

Neutralization plaque assays

Neutralization assays without complement were carried out in microtiter plates. Twenty-five µl of two-fold serial dilutions of antisera were mixed with 25 µl of a viral suspension containing 20 pfu (plaque forming units) of RSV. The neutralization reaction was allowed to proceed for 4 h at 4 °C. The virus-serum mixtures were added in plates containing HEp-2 cell sheets and then 150 µl of 1% methylcellulose was added as semi-solid medium. The plates were finally incubated for 4 days in a CO_2 incubator: titers were expressed as the highest dilution that neutralized 50% of the infectious units.

Blocking of neutralization by peptides

Two-fold serial dilutions of peptides (5, 2.5, and 1.25 µg/25 µl) were incubated overnight at 4 °C with 25 µl of monoclonal antibody diluted to 100% neutralizing activity. The plaque reduction assay was then carried out as described above. In these conditions, blocking activity was defined as the inhibition of the neutralizing activity of MAb and percentages were calculated from the number of plaques counted per dilution over untreated positive viral control.

Interleukin-2 assays

Splenocytes from either untreated or RSV-primed BALB/c mice (A_2 strain, 3 weeks after the second intraperitoneal injection) were cultured in flat-bottomed microtiter plates, using 2×10^5 cells/well and RPMI-1640 containing 10% fetal calf serum, 1% gentamycin and 50 µM β-mercaptoethanol as medium. Splenocytes were stimulated again in vitro in quadruplicate using either 0.1 m.o.i. of Long strain RSV or 10, 1, 0.1, or 0.01 µg of synthetic peptide. Microtiter plates were then incubated 4 days at 37 °C, in 5% CO_2 after which aliquots of the supernatants were harvested and frozen at -20 °C.

An IL-2 dependent cell line (CTLL-2; ATCC TIB 214) was used as previously described [9, 10] to determine the concentration of IL-2 in the samples. Briefly, two-fold dilutions of supernatants were prepared in quadruplicate in flat-bottomed microtiter plates and then 50 μl of a 1.5 × 10^5 cell/ml suspension of CTLL-2 were added. Plates were incubated at 37 °C for 24 h. Cell growth was monitored using the colorimetric MTT (tetrazolium) assay [11]. Ten μl of a filtered solution containing 5 mg of MTT/ml in PBS were added and the incubation was continued for 6 h. The dark blue crystals formed during the reaction were dissolved by vigorous shaking upon the addition of 100 μl of 0.04 N HCl prepared in isopropanol. The optical densities were read within an hour with a spectrophotometer (STL-Labinstruments, Austria) at 570 nm, using a reference wavelength of 630 nm. Finally, the IL-2 activity was calculated and expressed in units/ml [10]; commercial IL-2 (Rat T-cell polyclone, Collaborative Research Inc., Bedford, Mass., U.S.A.) was used as standard.

Immunization with peptides

Three intraperitoneal injections (21 days apart) of 5–10 μg of peptides coupled to KLH were given to groups of five BALB/c mice in the presence of either Freund's complete (the first injection) or incomplete adjuvant. A control group received KLH in adjuvant only. Fourteen days following the last injection, animals were bled and their sera analyzed by neutralization (see above) and ELISA assays against BSA coupled peptides and RSV. In ELISA assays, 1 ng/well of peptide coupled to BSA was adsorbed onto microtiter plates, followed by a treatment with 1.5% (w/v) BSA. Two-fold dilutions of serum were then added and the plates incubated for 2 h at 20 °C. After washing, wells were incubated with affinity-purified peroxydase-labeled anti-mouse IgG (Miles Laboratories, Elkhart, Ind., U.S.A.), washed again and revealed with O-phenylenediamine (0.4 mg/ml of 0.1 M citrate buffer pH 5 containing 0.03% H_2O_2). Titers were expressed as the last dilution giving an optical density 2 fold above background.

In protection studies, BALB/c mice were immunized intraperitoneally as above. Fourteen days after the last immunization, animals were challenged intranasally with $10^{4.2}$ pfu of RSV strain A2. Five days later, the animals were sacrificed and lungs tested for the presence of virus by plaque assays as previously described [18].

Selection of peptides

The hydropathicity profile of the fusion protein sequence (strain A2, 3) was plotted using the Kyte and Doolittle algorithm with a window set of nine amino acids. The analysis was carried out with the MacGene Plus program (Applied Genetic Technology, Inc., Fairview Park, Ohio). The profile obtained is reproduced in Fig. 1.

Synthesis of peptides

The sequences of selected peptides are given in Table 1. To facilitate the coupling of peptides to carrier proteins (see below), a cysteine residue was added at the N-termini of peptides
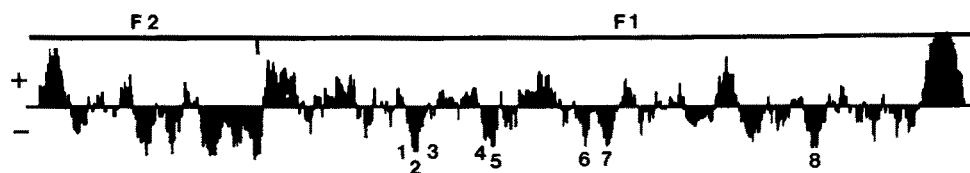

Fig. 1. Hydropathicity profile of the RSV fusion protein. The profile was obtained from the polypeptide sequence of the A2 strain (3), as calculated with the MacGene Plus program using the Kyte and Doolittle algorithm with a window set at nine residues. F2 and F1 polypeptide subunits are shown and the location of selected peptides

Table 1. Selected synthetic peptides corresponding to the major hydrophilic regions of the RS (A 2 strain) fusion protein

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1 (212)Cys-Ser-Ile-Ser-Asn-Ile-Glu-Thr-Val-Ile-Glu(222)
2 Cys-(216)Asn-Ile-Glu-Thr-Val-Ile-Glu-Phe-*Gln-Gln-Lys-Asn-Asn*-Arg-Leu-Leu-Glu(232)
3 Cys-(221)Ile-Glu-Phe-*Gln-Gln-Lys-Asn-Asn*-Arg-Leu-Leu-Glu-Ile-Thr-Arg-Glu-Phe(237)
4 (265)Pro-Ile-Thr-Asn-Asp-Gln-Lys-Lys-Leu-Met-Ser-Asn-Asn(277)-Gly-Cys
5 (275)Ser-*Asn-Asn*-Val-*Gln*-Ile-Val-Arg-*Gln-Gln*-Ser-Tyr-Ser-Ile(288)-Gly-Cys
6 Lys-Gly-(321)Leu-Cys-Thr-Thr-Asn-Thr-Lys-Glu-Gly-Ser-Asn-Ile-Cys-Leu(334)
7 Lys-Gly-(332)Ile-Cys-Leu-Thr-Arg-Thr-Asp-Arg-Gly-Trp-Phe-Cys-Asp(344)
8 Cys-(457)Tyr-Tyr-Val-Asn-Lys-Gln-Glu-Gly-Lys-Ser-Leu-Tyr-Val-Lys-Gly(471)

In italics, regions that show sequences shared by several peptides

2, 3, and 8, and a Gly-Cys pair was added to the C-terminus of peptides 4 and 5. A Lys-Gly pair was also added at the N-termini of the cyclic peptides 6 and 7. Peptides were synthesized on a p-alkoxybenzyl alcohol resin using N-9-fluorenylmethoxycarbonyl-amino acid derivatives as described [20]. Briefly, each amino acid was coupled by the dicyclo-hexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBT) method. Completeness of coupling was monitored by a ninhydrin test. The protecting groups were tert-butyl for Glu, Ser and Thr and tert-butyloxycarbonyl, tosyl and p-methoxybenzyl for Lys, Arg, and Cys residues, respectively. Between each coupling, the N-9-fluoro-methoxycarbonyl group was cleaved with 20% piperidine in N,N-dimethylformamide. Cleavage of the peptide from the resin and removal of the protecting groups was carried out by treatment with 55% tri-fluoroacetic acid in methylene chloride in the presence of 5% anisole. The remaining protecting groups were cleaved by treatment for 60 min at 0 °C in the presence of 5% anisole and 5% ethanedithiol as scavengers. The peptides were purified to 95% homogeneity by reverse-phase HPLC. The composition of the synthetic peptides was confirmed by amino acid analysis.

Coupling of peptides

Peptides 2, 3, 4, 5, and 8 were coupled either to keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) through the cysteine residue, using sulphosuccinimidyl 4-(p-maleimidophenyl) butyrate (Pierce Chemical Co., Rockford, Ill., U.S.A.) [7]. The peptides 6 and 7, which were naturally cyclic due to the formation of a disulfide group between the two cysteine residues, were conjugated to carrier polypeptides by treating with glutaraldehyde.

Results

Development of an efficient subunit vaccine against RSV requires the precise identification of humoral (B) and cellular (T-cell) epitopes of the viral proteins that are involved in protection. Based upon the hydropathicity profile of the F_1 subunit of the fusion protein (Fig. 1), eight peptides comprising from 10 to 17 amino acid residues of the F polypeptide were selected (Table 1). The ability of these peptides to stimulate murine B and T cells and also to confer protection was tested.

Immune responses to the peptides

In immunogenicity studies in BALB/c mice, peptides were conjugated to KLH to increase the immune responses; only peptides 1, 2, 3, 4, 7, and 8 could be solubilized efficiently and hence tested in vivo. Conjugated peptides 2, 3, 4, 7, and 8 stimulated B-cell activity as demonstrated by the production of specific anti-peptide antibodies detected in ELISA assays against BSA coupled homologous peptide using as antigen (Table 2). Titers obtained ranged from 1/100,000 to 1/400,000, that is 33–130 times that obtained with the KLH control. In contrast, peptide 1 was negative by ELISA. However, immune sera failed to react when RSV was substituted for the peptide antigens in the ELISA test. Nevertheless, immune sera obtained with peptide 3 contained neutralizing antibodies of the moderate but significant titer of 1/16 (Table 2). This result demonstrates that the antibodies induced could recognize a specific site on the fusion protein of RSV.

The protection conferred by the synthetic experimental vaccines 2, 3, 4, 7, and 8 was tested by intranasal challenge of immunized mice with the A2 strain of RSV (Table 2). No significant reduction of viral titers was observed in the lungs in comparison with controls, which suggests that the regions used were not or only partially involved in protective immunity or that an inadequate level of immunity was induced in the host.

The ability of the peptides to be recognized in vitro by T-cells stimulated in vivo with RSV was tested by determining the production of interleukin-2 (IL-2): the different peptides and splenocytes isolated from either untreated of RSV-immunized (Long strain) BALB/c mice were incubated together (Fig. 2). The production of IL-2 in splenocytes from BALB/c mice previously immunized with the virus was increased in comparison with untreated mice. In the latter case, no effect was observed when the peptides were used to stimulate splenocytes

Table 2. Immunogenicity of peptides coupled to KLH: ELISA, 50% neutralizing (N), and protection assays

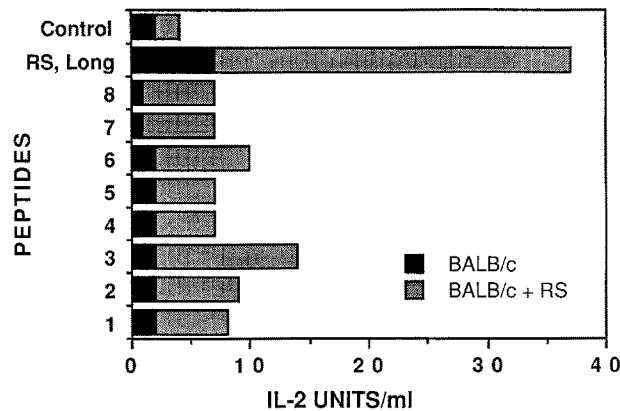| Peptide	Location	ELISA	50% N	pfu/g of lung $(\log_{10})$
1	Cys 212-Glu 222	neg	neg	nt
2	Asn 216-Glu 232	1/102,400	neg	4.4 ± 0.1
3	Ile 221-Phe 237	1/204,800	1/16	4.4 ± 0.1
4	Pro 265-Asn 277	1/409,800	neg	4.5 ± 0.2
7	Ile 332-Asp 344	1/409,800	neg	4.5 ± 0.4
8	Tyr 457-Gly 471	1/102,400	neg	4.8 ± 0.2
KLH control		1/3,200	neg	4.8 ± 0.2
BSA control		1/200	neg	4.8 ± 0.15

neg Negative
nt Not tested

Fig. 2. Interleukin-2 (IL-2) concentrations obtained after in vitro stimulation of either RSV-immunized or untreated BALB/c splenocytes. Splenocytes were restimulated either with 0.1 μg of peptides 1 to 8 or with 0.1 m.o.i. of RSV (Long strain). The control corresponded to the basal production of IL-2 in vitro of unstimulated splenocytes from untreated or immunized mice

since less than 2 units/ml of IL-2 were produced in these assays as well as in the negative control (splenocytes non-stimulated in vitro). However, the use of the peptides to stimulate splenocytes isolated from RSV-immunized mice was effective and variable according to the peptide used: 14.0 ± 1.5 units/ml of IL-2 were produced with peptide 3, 10.0 ± 3 with peptide 6, 9.0 ± 2.6 with peptide 2, and in the range of 7.0 ± 1.9 to 8.0 ± 2.8 units/ml with peptides 1, 4, 5, 7, and 8. In these assays, the level of IL-2 produced was significant (t-test) compared with the basic level of 4.2 ± 0.5 units/ml of the control and with the maximum level attained of 37.5 ± 8 units/ml with splenocytes stimulated in vitro with 0.1 m.o.i. of RSV.

The reactivity of the eight synthetic peptides was then examined by analysis with four monoclonal antibodies (MAbs) with high neutralizing activity, directed against the fusion protein of RSV. These MAbs were identified by immunoprecipitation and subsequent SDS-PAGE analysis.

Characterization and relationships of MAbs 7C2, 19, B4, and 11

Table 3 shows that MAbs 7C2, 19, B4, and 11 efficiently neutralized both the human Long and the bovine A-51908 strains of RSV. The degree of neutralization varied as follows: 7C2 > 19 > B4 > 11, indicating differences among these MAbs. When the four MAbs were used to passively immunize BALB/c mice, significant reduction of virus titers were found in lungs upon challenge as compared with the non-immunized control mice, indicating that they were capable of conferring protection.

Competitive ELISA assays were carried out to define the relationship that could exist among the specific epitopes of the MAbs. The variations observed in the level of competition obtained using MAbs B4, 11, and 19 against MAb 7C2

Table 3. 50% Neutralization activities and passive protection conferred in BALB/c mice by MAbs

MAbs	Human RSV[a] (long)	Bovine RSV[a] (A-51908)	Virus titer[b]
7C2	1/17,000	1/17,000	<1.7
B4	1/1,024	1/4,096	<1.7
11	1/512	1/1,024	<1.7
19	1/8,000	1/17,000	<1.7
Control	–	–	5.0±0.3

[a] 50% inhibition titer of MAbs (ascites)
[b] Titer of virus expressed in $\log_{10}$ pfu/g of lungs
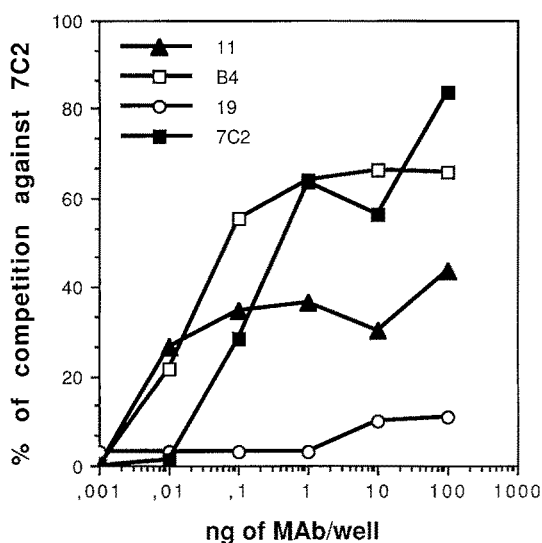
Fig. 3. Competitive ELISA assays of MAbs 7C2, B4, 11, and 19 with MAb 7C2

indicated the presence of three epitopes (Fig. 3). The epitopes recognized by MAbs B4 and 7C2 were probably very similar due to a nearly equivalent competition attained with these monoclonal antibodies; this epitope was named epitope 1. A second epitope was recognized by MAb 11 which competed 40% with MAb 7C2. This result suggested a partial overlapping of epitopes 1 and 2. Finally, a third epitope was identified with MAb 19 which showed less than 10% competition with MAb 7C2.

Recognition of peptides by MAbs

The possibility that the synthetic peptides be recognized by the above MAbs was tested by measuring their ability to block the neutralization propriety of the different MAbs. Table 4 shows that peptide 2 efficiently inhibited 75–100% of the neutralization capacity of MAbs 7C2, 11, and 19 and to a lesser degree that of MAb B4. Peptide 3 completely inhibited MAbs 7C2 and 19 while it

Table 4. Blocking of MAb neutralization by peptides

MAbs	Peptides							
	1	2	3	4	5	6	7	8
Epitope 1								
7C2	−	+ + + +	+ + + +	−	+ + + +	−	−	−
B4	−	+	+ +	−	+	−	−	−
Epitope 2								
11	−	+ + + +	+ +	−	−	−	−	−
Epitope 3								
19	−	+ + + +	+ + + +	−	+ +	−	−	−

Inhibition of neutralization: + + + + 75–100%, + + + 50–75%, + + 25–50%, + 0–25%, − 0%

moderately inhibited MAbs B4 and 11. Finally, peptide 5 inhibited neutralization by MAbs 7C2, 19, and B4, in decreasing order. No other peptide inhibited the MAbs used.

The specificity of the reaction between peptides and MAbs was further confirmed in dot-blot assays. In these experiments, all four MAbs were found only to react positively with the peptides 2, 3, and 5 (data not shown).

Discussion

Eight peptides have been selected on the basis of their correspondence to the major hydrophilic regions of the fusion protein of the RSV. Peptides 2, 3, 4, 7, and 8 conjugated to KLH, demonstrated an immunogenic potential since they efficiently induced B-cell activity. Immune sera reacted to a high degree with their respective peptide antigen in ELISA but failed to react with the RSV containing the "native" fusion protein antigen. This lack of reactivity suggests that the epitopes involved on the fusion polypeptide were inaccessible to the anti-peptide antibodies. This could be explained by the hypothesis that the protein regions predicted to be hydrophilic were either unexposed or masked by other viral polypeptides.

Conjugated peptide 3, which contains the region Ile221-Phe237 of the fusion polypeptide, was the only of the 6 peptides tested in immunization assays to induce a low level of neutralizing antibodies, even though no protection was conferred. The neutralizing activity of the immune serum clearly demonstrated the immunogenicity of the epitope involved and confirmed our previous localization of a major neutralization site of the fusion protein of RSV in the region Ile221-Glu232 [20]. Peptide 2, however, also contained this latter region, being composed of the sequence Asn216-Glu232, but did not induce neutralizing antibodies. This result could be explained either by differences in the coupling

yield or by the hypothesis that the 232–237 region contributes to the neutralization epitope.

All the selected peptides induced the in vitro production of IL-2 in splenocytes previously stimulated in vivo with RSV, with peptides 3, 6, and 2 being the most efficient. If the concentrations of IL-2 produced in assays with stimulated splenocytes are corrected for those obtained with non-stimulated splenocytes, the quantity of IL-2 produced upon in vitro stimulation with peptides 3, 6, and 2 attained 40, 27, and 23%, respectively, of the level obtained with the positive RSV control. Consequently, stimulation with the peptides was quite significant and efficient considering the fact that each peptide only contained a single stimulating site whereas whole virus comprises several. These experiments demonstrated specific recognition of the peptides by primed T-cells.

Finally, the ability of the eight peptides to block the neutralization activity of MAbs 7C2, B4, 11, and 19 was determined. Neutralization of the four MAbs examined was inhibited by peptides 2, 3, and 5 only, except MAb 11 which did not react with peptide 5. Also, the four MAbs were specific for these three peptides in dot blot assays. Peptides 2 and 3 were close overlaps containing the regions Asn216-Glu232 and Ile221-Phe237, respectively, of the fusion protein. Reactivity of these peptides with the closely related (see below) MAbs 7C2 and B4 and also with the related Mab 11 was expected since both sequences contained the protein region Ile-221-Glu232 previously identified as a major neutralization site specific to MAb 7C2 [20, 23] (see below). Curiously, peptide 5, comprised of the fusion protein region Ser275-Ile288, also reacted positively with the MAbs 7C2 and B4. Analysis of the sequences involved revealed that the three peptides included two consecutive Gln and Asn residues. We hypothesize that these four residues could be important in promoting interaction between peptides and MAbs 7C2 and B4. The fact that the Gln and Asn pairs were in reverse orientation in one peptide would probably not affect complex formation due to the extensive structural homology between the two amino acids (Asn and Gln are both uncharged and hydrophilic and contain an amide group which is connected to the α carbon atom by a methyl and dimethyl group, respectively). At present, this is the most likely explanation even though the Gln and Asn pairs were separated by either one or five amino acid residues. Indeed, Lopez et al. [8] have already demonstrated the critical importance of Asn residues at positions 262 and 268 of the fusion protein: point mutations at either position allowed virus to escape neutralization by this group's neutralizing MAb 47F. Both results clearly suggest that region 262–288 could play an important role in protective immunity against RSV. Furthermore, the ability of the peptides 2, 3, and 5 to block neutralization by MAbs 7C2 and B4 suggests that a relationship exists between the 221–232 (common positions within peptides 2 and 3) and 275–288 regions. For example, a discontinuous antigenic site may be formed by these two regions. Further studies are under way to confirm our hypothesis.

Neutralization by MAb 19 was inhibited by peptides 2, 3, and 5, even though

this antibody was found to be different from MAb 7C2 (see below): it is not excluded that their specificity could partially overlap. At present, the significance of this result is unclear. We postulate that residues common to the three peptides could mimic a part of the specific MAb 19 epitope.

Evidence of an antigenic determinant composed of 2 epitopes

Highly neutralizing MAbs 7C2, B4, 11, and 19 were used in competitive ELISA assays against MAb 7C2. The comparable levels of competition observed in assays using MAbs B4 and 7C2 against MAb 7C2 indicated a close relationship or identity between these antibodies and suggested that the same epitope (identified as epitope #1) was responsible for their induction. Nevertheless, these two MAbs were distinguishable by their neutralizing activities as well as by their degree of reactivity with synthetic peptides 2, 3, and 5. In competition assays, MAb 11 inhibited MAb 7C2 by 40%. By analogy, a similar range would have probably been attained with MAb B4. The level of competition obtained suggested that the specific epitope (epitope #2) detected by MAb B4 overlaps or is identical with the MAb 7C2's epitope. Interestingly, we had previously reported the presence of four distinct antigenic sites on the fusion protein of RSV [23]: antigenic site A reacted specifically with the highly neutralizing MAb 7C2 and as such, was identified as the major neutralization site corresponding to epitopes #1 and 2 whereas sites B and C were secondary neutralization sites and site D was not involved in neutralization. The major neutralization epitope A was located at or around the region extending from Ile221 to Glu232 of the F_1 subunit of the fusion protein [20, 23]. Results obtained here in ELISA competition assays with MAb 11, B4, and 7C2 suggested that two overlapping epitopes constituting a single antigenic determinant had induced the three related monoclonal antibodies. In contrast, MAb 19 only inhibited MAb 7C2 by 10%. This result was considered non-significant and suggested the presence of an independent epitope, identified as #3. Based on the neutralization activities of MAb 19, it is probable that epitope #3 did not correspond to the previously identified antigenic sites B, C, and D; if this is the case, MAb 19 would have detected a fifth antigenic site. The relationship between the epitopes of MAb 7C2, B4, and 11 as well as the independence of the epitope of MAb 19 were confirmed with MAb-resistant viral mutants (unpubl. results).

Other important characteristics of the four MAbs examined here were their ability to neutralize both the human Long and bovine A-51908 strains and to protect mice from infection. We have also found that all four MAbs could inhibit the fusion of RSV-infected cells in vitro (unpubl. results; [5]); thus, three and maybe more fusion inhibiting sites could be contained in the fusion protein of RSV. A study conducted by Walsh [25] has also resulted in the identification of at least two distinct fusion inhibiting antigenic sites of the F polypeptide. Other nonfusion-inhibiting antigenic sites have also been reported [2, 25].

Acknowledgements

References

1. Anderson LJ, Bingham P, Hierholzer JC (1988) Neutralization of respiratory syncytial virus by individual and mixtures of F and G protein monoclonal antibodies. J Virol 62: 4232–4238

2. Anderson LJ, Hierholzer JC, Stone YO, Tsou C, Fernie BF (1986) Identification of epitopes on respiratory syncytial virus proteins by competitive binding immunoassay. J Clin Microbiol 23: 475–480

3. Elango N, Satake M, Coligan JE, Norrby E, Camargo E, Venkatesan S (1985) Respiratory syncytial virus fusion glycoprotein: nucleotide sequence of mRNA, identification of cleavage activation site and amino acid sequence of the N-terminus of F1 subunit. Nucleic Acids Res 13: 1559–1574

4. Giminez HB, Cash P, Melvin WT (1984) Monoclonal antibodies to human respiratory syncytial virus and their use in comparison of different isolates. J Gen Virol 65: 963–971

5. Kennedy HE, Jones BV, Tucker EM, Ford NJ, Clarke SW, Furze J, Thomas LH, Stott EJ (1988) Production and characterization of bovine monoclonal antibodies to respiratory syncytial virus. J Gen Virol 69: 3023–3032

6. Lehmkhul HD, Smith MH, Cutlip RC (1980) Morphogenesis and structure of caprine respiratory syncytial virus. Arch Virol 65: 269–276

7. Liu FT, Zinnecker M, Hamaoka T, Katz DH (1979) New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. Biochemistry 18: 690–697

8. Lopez JA, Penas C, Garcia-Barreno B, Melero JA, Portela A (1990) Location of a highly conserved neutralizing epitope in the F glycoprotein of human respiratory syncytial virus. J Virol 64: 927–930

9. Mansour S, Thibodeau L, Perrin P, Sureau P, Mercier G, Joffret ML, Oth D (1988) Enhancement of antigen-specific interleukin-2 production by adding liposomes to rabies antigens for priming. Immunol Lett 18: 33–66

10. Micusan V, Mercier G, Bhatti AR, Reiser RF, Bergdoll MS, Oth D (1986) Production of human and murine interleukin-2 by toxic shock syndrome toxin-1. Immunology 58: 203–208

11. Mossman T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. J Immunol 65: 55–63

12. Olmsted RA, Elango N, Prince GA, Murphy BR, Johnson PR, Moss B, Chanock RM, Collins PL (1986) Expression of the F glycoprotein of respiratory syncytial virus by a recombinant vaccinia virus: Comparison of the individual contributions of the F and G glycoproteins to host immunity. Proc Natl Acad Sci USA 83: 7462–7466

13. Örvell C, Norrby E, Mufson MA (1987) Preparation and characterization of monoclonal antibodies directed against five structural components of human respiratory syncytial virus subgroup B. J Gen Virol 68: 3125–3136

14. Routledge EG, McQuillan J, Samson ACR, Toms GL (1985) The development of monoclonal antibodies to respiratory syncytial virus and their use in diagnosis by indirect immunofluorescence. J Med Virol 15: 305–320

15. Scopes GE, Watt PJ, Lambden PR (1990) Identification of a linear epitope on the fusion glycoprotein of respiratory syncytial virus. J Gen Virol 71: 53–59

16. Stott EJ, Bew MH, Taylor G, Jebbett J, Collins AP (1984) The characterization and uses of monoclonal antibodies to respiratory syncytial virus. Dev Biol Standard 57: 237–244

17. Stott EJ, Taylor G (1985) Respiratory syncytial virus. Brief review. Arch Virol 84: 1–52

18. Taylor G, Stott EJ, Fernie BF, Cote PJ, Collins AP, Hughes M, Jebbett J (1984a) Monoclonal antibodies that protect against respiratory syncytial virus infection in mice. Immunology 52: 137–142

19. Taylor G, Stott EJ, Hughes M, Collins AP (1984b) Respiratory syncytial virus infection in mice. Infect Immun 43: 649–655

20. Trudel M, Nadon F, Séguin C, Dionne G, Lacroix M (1987a) Identification of a synthetic peptide as part of a major neutralization epitope of repiratory syncytial virus. J Gen Virol 68: 2273–2280

21. Trudel M, Nadon F, Séguin C, Ghoubril S, Payment P, Trépanier P (1986) Immunovirological studies on human respiratory syncytial virus structural proteins. Can J Microbiol 32: 15–21

22. Trudel M, Nadon F, Séguin C, Simard C, Lussier G (1989) Experimental polyvalent ISCOMS subunit vaccine induces antibodies that neutralize human and bovine respiratory syncytial virus. Vaccine 7: 12–16

23. Trudel M, Nadon F, Séguin C, Payment P, Talbot PJ (1987b) Respiratory syncytial virus fusion glycoprotein: further characterization of a major epitope involved in virus neutralization. Can J Microbiol 33: 933–938

24. Walsh EE, Hruska J (1983) Monoclonal antibodies to respiratory syncytial virus proteins: identification of the fusion protein. J Virol 47: 171–177

25. Walsh EE, Cote PJ, Fernie BF, Schlesinger JJ, Brandriss MW (1986) Analysis of the respiratory syncytial virus fusion protein using monoclonal and polyclonal antibodies. J Gen Virol 67: 505–513

26. Walsh EE, Hall CB, Schlesinger JJ, Brandriss MW, Hildreth S, Paradiso P (1989) Comparison of antigenic sites of subtype-specific respiratory syncytial virus attachment proteins. J Gen Virol 70: 2953–2961

27. Williams AF, Galfre G, Milstein C (1977) Analysis of cell surfaces by xenogeneic myeloma hybrid antibodies: differentiation antigens of rat lymphocytes. Cell 12: 663–673

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