

Fine specificity of the antibody response to a synthetic peptide from the fusion protein and protection against measles virus-induced encephalitis in a mouse model

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A synthetic peptide representing residues 397–420 from the measles virus (MV) fusion (F) protein was tested for its structure, immunogenicity and protective capacity against intracerebral challenge with a neuroadapted strain of MV. Analysis of the peptide by mass spectrometry showed that it was linear, despite the presence of two cysteine residues in the sequence. Circular dichroism spectroscopy highlighted a weak preference for the peptide to adopt an α -helical conformation. The peptide was shown to be immunogenic in BALB/c and C57BL/6 mice after intraperitoneal immunization in Freund's adjuvant, and anti-peptide antibodies from both strains of mice reacted with the MV as a solid phase antigen on an ELISA plate. When the fine specificity of the anti-peptide antibody response was

examined using overlapping 8-mer peptides, serum antibodies from BALB/c mice recognized the region between residues 407–417 whereas antibodies from C57BL/6 mice recognized the region 408–420 of the 397–420 peptide sequence. Although anti-397–420 antibodies had no demonstrable neutralizing activity, protection against challenge with a neuroadapted strain of MV was demonstrated following active immunization with peptide in C57BL/6 mice or after passive transfer of anti-peptide antibodies in BALB/c mice. These findings highlight the importance of the 397–420 region in the induction of protective antibodies in the MV encephalitis mouse model, and suggest that this epitope might be a good candidate for inclusion in a future MV synthetic peptide vaccine.

Introduction

Measles virus (MV), a member of the family *Paramyxoviridae*, is responsible for an acute febrile illness of early childhood, and although there is widespread vaccination coverage with a live attenuated vaccine, the virus still remains responsible for over one million deaths per year in developing countries. This is mainly attributed to socio-economic factors such as poor nutritional status, lack of supportive health care, general poor resistance to infection as well as thermal lability of the live measles vaccines and the presence of maternal antibodies which interfere with the vaccine virus. Thus, there is an urgent need for the development of a new stable vaccine capable of inducing protective immunity in early childhood.

Recent advances in immunology and peptide chemistry have allowed the development of subunit vaccines, based on

peptides representing protective B- and T-cell antigenic sites. Although a peptide vaccine is not currently being used for mass vaccination, results in experimental animal models have highlighted the potential of the peptide approach in successfully inducing protection against various pathogens (Meloan *et al.*, 1995). In the case of MV, surface glycoproteins F and H play an important role in immunity (Merz *et al.*, 1980; Varsanyi *et al.*, 1984; Wild *et al.*, 1992), and studies have shown that protection of young mice against challenge with a neuroadapted strain of MV can be achieved by means of immunization with synthetic peptides representing mimics of conformational (Steward *et al.*, 1995) or linear B-cell epitopes (Obeid *et al.*, 1995) from the F or H (Ziegler *et al.*, 1996) glycoproteins of MV.

In the present study, we examined the structure and tested the immunogenicity of a synthetic peptide representing residues 397–420 from the F protein of MV in two inbred strains of mice, and analysed the fine specificity of the antibody response. The sequence 397–420 was selected on the basis of

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(i) prediction methods for surface exposure (Partidos *et al.*, 1991), (ii) it contains the region 404–414 previously identified as a B-cell epitope (Partidos *et al.*, 1991) and shown to confer protection in the mouse MV-induced encephalitis model when linked to two copies of a T-helper epitope (Obeid *et al.*, 1995), and (iii) this region from the F protein of the related Sendai virus contains a neutralizing epitope (Portner *et al.*, 1987). In addition, the protective capacity of 397–420 peptide was tested against challenge with a neuroadapted strain of MV in C57BL/6 mice and BALB/c mice.

Methods

■ **Peptide synthesis.** Synthetic peptides representing residues 397–420 (C-Y-T-T-G-T-I-I-N-Q-D-P-D-K-I-L-T-Y-I-A-A-D-H-C) and 404–414 (I-N-Q-D-P-D-K-I-L-T-Y) from the fusion protein of MV were synthesized using Fmoc chemistry. Briefly, peptide synthesis was performed by converting Fmoc protected amino acids to the hydroxy-benzotriazol activated esters through treatment with hydroxy-benzotriazol and diisopropylcarbodiimide in dimethyl formamide (DMF). The subsequent coupling reactions were performed in DMF, and the Fmoc groups were removed with 20% piperidine in DMF, followed by a series of washes in DMF. After synthesis, side-chain protecting groups were removed and the peptide was cleaved from the resin with trifluoroacetic acid in the presence of scavengers. After cleavage, peptides were extracted into diethyl ether and purified by reverse phase C18 semi-preparative high performance liquid chromatography (HPLC) using a two solvent system: solvent A, 0.1% trifluoroacetic acid (TFA) in water; solvent B, 0.1% TFA in acetonitrile. The peptide was eluted using a 30 min linear gradient of B (25–33%). In addition, a series of 8-mer peptides (overlapping by one residue) covering the peptide sequence 397–420 (Fig. 1) was synthesized on a derivatized membrane. The Fmoc protected amino acids were provided within a commercially available package (SPOTs Kit, Genosys) which included computer software for the determination of a protocol for coupling reactions.

■ **Circular dichroism.** Stock solutions of HPLC purified peptide were prepared at 0.14 mM in distilled water. The concentration was calculated from the absorbance at 274.4 nm using ϵ (molar extinction coefficient) = 2840 M⁻¹ cm⁻¹ for the two Tyr chromophores. Circular dichroism (CD) spectra were recorded at 22 °C on a Jobin–Yvon IV autodichrograph using 0.1 cm path-length to keep absorbance values below 1 from 185 to 270 nm. Optical activities were reported as ellipticity per peptidic bond (θ , deg.cm².dmol⁻¹). Calculation of the α -helical content was performed according to the formula based on a 26 protein data set (Zhong & Johnson, 1992): % α -helix = $\theta_{222}/3298 \times (-10)$.

■ **Virus.** A rodent neuroadapted strain of MV (CAM/RBH strain; kindly provided by Uve Liebert, Germany (Liebert & ter Meulen, 1987) was used in this study. Stocks of virus were prepared by passage in suckling mouse brains. Mouse brain homogenates (25%) from infected suckling mice were used as a source of virus and were stored at –70 °C. For the neutralization assays, the Edmonston strain of MV grown in Vero cells was used.

■ **Cells.** Vero cells, a continuous cell line of African Green Monkey kidney were used within ten passages of a cloned mycoplasma-free stock and stored in liquid nitrogen at passage 15. Cells were grown in sterile tissue culture flasks at 37 °C, in a 5% CO₂ atmosphere using 20 ml of 199 medium supplemented with 5% FCS, 4.5% (v/v) sodium bicarbonate, streptomycin (100 µg/ml) and penicillin (100 units/ml).

■ **Immunization.** Groups of four to five female, 6–8-week-old BALB/c and C57BL/6 inbred strains of mice (purchased from the National Institute of Medical Research, Mill Hill, UK) were used in this study. Mice were immunized intraperitoneally (i.p.) with 100 µg of the 397–420 peptide emulsified in Freund's complete adjuvant (FCA) (1:1) and 7 weeks later they received a booster i.p. injection of the same dose in Incomplete Freund's adjuvant (IFA) (1:1). Mice were bled at weekly intervals and serum samples were stored at –20 °C. At the end of the experiment (17 weeks after priming), mice were killed and pooled splenocytes from each group were tested for proliferative responses (as described previously; Partidos *et al.*, 1991) upon *in vitro* restimulation with various concentrations of 397–420 peptide or heat inactivated MV.

For protection studies, groups of 3-week-old C57BL/6 female mice (eight per group), were immunized i.p. with 100 µg of 397–420 peptide emulsified in FCA (1:1) and boosted i.p. 2 weeks later with 50 µg of peptide in an IFA (1:1) emulsion. The following day mice were challenged intracranially (i.c.) with 10⁴ p.f.u. (in a 25 µl volume) of a neuroadapted strain of MV. Mice were monitored daily for the development of clinical signs and percentage mortality was assessed over a period of 1 month.

For the passive transfer experiments, groups of 2-week-old BALB/c mice (six per group) received 200 µl of anti-397–420 peptide serum (titre of log₁₀ 4) or normal mouse serum i.p.; 24 h later they were challenged i.c. with 10⁴ p.f.u. of neuroadapted strain of MV in a 25 µl volume. Mice were monitored daily for the development of clinical signs and percentage mortality was assessed over 25 days.

■ **ELISA for detection of anti-peptide and anti-MV antibody responses.** Titres of antibodies against the peptides or MV were determined by an ELISA. Wells of microtitre plates were coated overnight at 4 °C with 50 µl per well of 5 µg/ml solution of peptide or MV (2.5 µg/ml sucrose gradient purified Edmonston strain of MV) in carbonate–bicarbonate buffer (0.1 M, pH 9.6). Plates were washed five times with tap-water prior to and following blocking with 1% gelatin in PBS for 2 h at 37 °C, dried and stored at 4 °C. Sera were titrated across the plate by serial twofold dilutions in diluent (PBS–0.25% gelatin–0.05% Tween 20) and incubated for 1 h at 37 °C. After washing with tap-water, 50 µl of rabbit anti-mouse IgG–peroxidase conjugate at a dilution of 1/1000 in diluent was pipetted into each well and the plates incubated as before. After washing, bound enzyme was detected by adding 50 µl per well of a chromogen solution (0.04% *o*-phenylenediamine + 0.004% hydrogen peroxide in 0.1 M citric acid–phosphate buffer pH 5.0). After 10 min, the colour reaction was stopped with 25 µl per well of 2 M sulphuric acid, and the absorbance at 492 nm was measured on a Titertek Multiskan (Flow Laboratories). Titres are expressed as log₁₀ of the reciprocal of the antibody dilution giving an absorbance greater than 0.2 at 492 nm. The ELISA SPOT assay was performed and developed according to the manufacturer's instructions (Genosys).

■ **Neutralization assay.** Serial twofold dilutions of anti-peptide serum were prepared in a sterile 96-well flat-bottomed tissue culture plate. Dilutions were made in 199 Earle's medium supplemented with 5% FCS, streptomycin (100 µg/ml) and penicillin (100 units/ml). To each well (excluding cell controls), 50 µl of MV at 50 p.f.u. per well was added, and plates were incubated at 37 °C for 90 min; 50 µl of a suspension of Vero cells (10⁶ cells/ml) was added to each well and plates were incubated for 3 h at 37 °C in 5% CO₂ atmosphere. Using a multichannel dispenser, medium was gently removed and 150 µl of CMC medium (87 ml CMC, 10 ml 10 × 199 medium, 1 ml FCS, 200 µl antibiotics; 100 µg/ml streptomycin and 100 units/ml penicillin) was added to each well. Plates were incubated for 4–6 days at 37 °C in a 5% CO₂ atmosphere, until cytopathic effect was observed.

Peptide	Sequence
P1 (397-404)	C-Y-T-T-G-T-I-I
P2 (398-405)	Y-T-T-G-T-I-I-N
P3 (399-406)	T-T-G-T-I-I-N-Q
P4 (400-407)	T-G-T-I-I-N-Q-D
P5 (401-408)	G-T-I-I-N-Q-D-P
P6 (402-409)	T-I-I-N-Q-D-P-D
P7 (403-410)	I-I-N-Q-D-P-D-K
P8 (404-411)	I-N-Q-D-P-D-K-I
P9 (405-412)	N-Q-D-P-D-K-I-L
P10 (406-413)	Q-D-P-D-K-I-L-T
P11 (407-414)	D-P-D-K-I-L-T-Y
P12 (408-415)	P-D-K-I-L-T-Y-I
P13 (409-416)	D-K-I-L-T-Y-I-A
P14 (410-417)	K-I-L-T-Y-I-A-A
P15 (411-418)	I-L-T-Y-I-A-A-D
P16 (412-419)	L-T-Y-I-A-A-D-H
P17 (413-420)	T-Y-I-A-A-D-H-C

Fig. 1. Octameric overlapping peptides synthesized on derivatized membrane.

Results

Characterization of the structure of 397–420 peptide

Crude 397–420 peptide was purified by HPLC and a major product was eluted with a retention time of 29 min. Analysis of the HPLC purified peptide by matrix assisted laser desorption ionization/time-of-flight (MALDI/TOF) mass spectrometry showed that the sulfhydryl groups of the two cysteines at positions 397 and 420 were not involved in an intermolecular disulfide bond as the experimental molecular mass of the peptide was demonstrated to be $[M+H]^+ : 2670.6$ which is consistent with the expected $[M+H]^+ : 2668.9$ molecular mass. The linearity of the 397–420 peptide was also confirmed by the Ellman's reagent DTNB [5,5 = 92-dithio bis(2-nitrobenzoate)] which allows the determination of free sulfhydryl groups (Ellman, 1959) (data not shown).

Analysis of the CD spectrum of the peptide recorded in pure water showed a minimum at 199 nm which is ascribed to the random coil structures (Fig. 2). In addition, the CD spectrum of 397–420 peptide was recorded in the presence of 50% trifluoroethanol (TFE), a solvent which stabilizes helices in peptides which already have an α -helical propensity (Rajan & Balaram, 1996). Fig. 2 shows the presence of double minima at

209 and 222 nm, characteristic of an α -helical structure. The α -helical content of the peptide was calculated to be 22%.

Kinetics of the antibody response to the 397–420 peptide in inbred strains of mice

The immunogenicity of the 397–420 peptide was assessed after intraperitoneal immunization of groups of BALB/c (H-2^d) and C57BL/6 (H-2^b) mice. Using an indirect ELISA, the reactivity of serum antibodies with peptide 397–420 and 404–414 as solid phase antigens was determined. As shown in Fig. 3, peptide 397–420 was immunogenic in both strains of mice. No differences in antibody titres between BALB/c and C57BL/6 mice were observed. Similar patterns of antibody responses were observed when serum samples were tested for reactivity with the 404–414 peptide (Fig. 3). However, these responses were lower as compared to the responses observed against the 397–420 peptide and BALB/c mice responded more strongly than the C57BL/6 mice to the 404–414 peptide.

Fine specificity of the antibody response to the 397–420 peptide

To determine the location of the B-cell site(s) within the 397–420 sequence, an ELISA SPOTs Kit was employed using

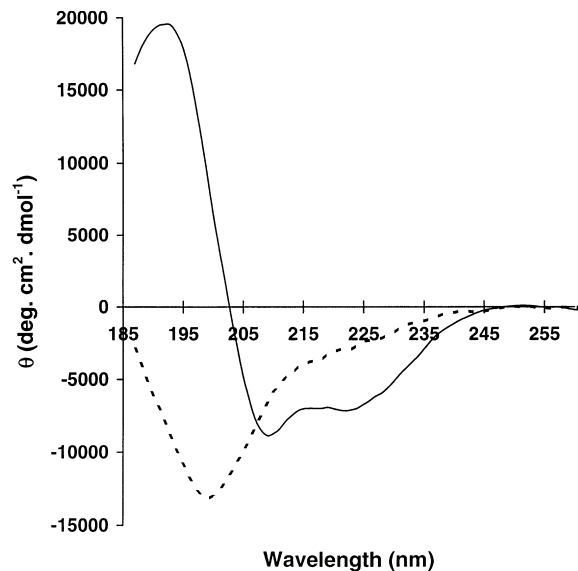


Fig. 2. CD spectra of the 397–420 peptide in water (---) or in the presence of 50% TFE (—). The concentration of peptide was 35 nM. The spectra were recorded at 22 °C and the path length was 0.1 cm.

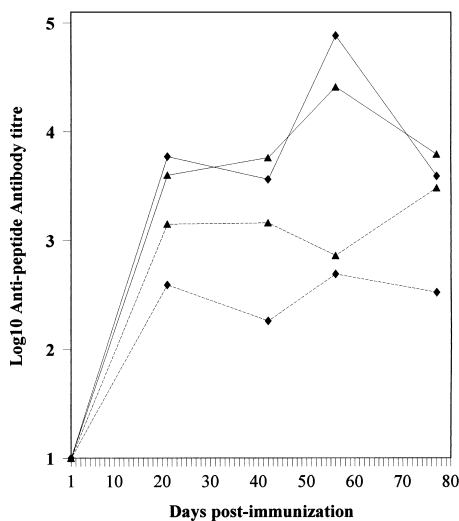


Fig. 3. Kinetics of the antibody response to the 397–420 (—) and 404–414 (---) peptides in BALB/c (◆) and C57BL/6 (▲) mice. Mice were immunized i.p. with 100 µg of the peptide emulsified in FCA and boosted 7 weeks later by the same route with 100 µg of peptide emulsified in FIA. Data are presented as means of antibody titre from a groups of four or five mice. Standard deviations were consistently less than 10%.

overlapping (by one residue) 8-mer peptides synthesized on a derivatized membrane. As shown in Fig. 4(a), anti-397–420 antibodies from BALB/c mice did not recognize peptides covering the region 397–406. However, a strong signal was observed in the region encompassing residues 407–417. Residues 418, 419 and 420 did not appear to be involved in the binding of anti-397–420 antibodies. Anti-397–420 antibodies raised in C57BL/6 mice reacted strongly with peptides

covering the region 408–420 whereas no reactivity was observed with the amino-terminal region 397–407 (Fig. 4b).

Reactivity of the anti-397–420 antibodies with MV

The reactivity of the anti-397–420 antibodies with MV was tested using an indirect ELISA with MV as a solid phase antigen. Serum samples from BALB/c and C57BL/6 mice immunized with 397–420 peptide reacted strongly with the MV ($\log_{10} 2.43 \pm 0.23$ and 2.33 ± 0.23 respectively). However, when serum samples from both strains of mice were tested for neutralizing activity, no significant reduction of plaque numbers was detected (data not shown).

Evaluation of the protective efficacy of 397–420 peptide against challenge with MV

The protective efficacy of the immune response to the 397–420 peptide induced following i.p. immunization was tested in C57BL/6 mice. Following i.c. challenge with neuroadapted MV, the peptide-immunized group showed a significant protection (Fisher's exact test; $P < 0.001$) as compared to the control group (PBS immunized mice) 30 days post-challenge (Fig. 5). Significant protection (Fisher's exact test; $P < 0.0150$) was also observed when anti-397–420 serum was passively administered in BALB/c mice 24 h prior to challenge with the neuroadapted strain of MV (Fig. 6).

Discussion

MV F protein is a surface glycoprotein and induces a protective immune response after infection. In previous studies, it has been demonstrated that a region represented by residues 404–414 is an important B-cell epitope. Anti-404–414 antibodies were shown to be reactive with MV in several strains of mice (Partidos *et al.*, 1991), and confer protection in a mouse encephalitis model after collinear synthesis at the carboxyl terminus of two copies of a T-helper epitope (Obeid *et al.*, 1995).

The data presented in this report indicate that the synthetic peptide 397–420 from MV F protein, comprising the sequence 404–414, can be immunogenic without the addition of any T-helper epitope. This suggests the presence of a T-helper epitope(s) in the peptide sequence. Indeed, splenocytes from both BALB/c and C57BL/6 mice were shown to proliferate upon *in vitro* restimulation with 10 µg peptide per culture (stimulation indices 2.37 and 5.5 respectively, as compared to the stimulation indices of 0.93 and 0.87 respectively for the control 397–404 peptide). However, no cross-reactivity with inactivated MV was observed within the range of antigen concentrations tested (data not shown) which suggests that the peptide might not be available after the processing of the virus.

In our previous studies we have shown that the lack of immunogenicity of the 404–414 peptide can be overcome by the addition of a T-helper epitope (Partidos *et al.*, 1991).

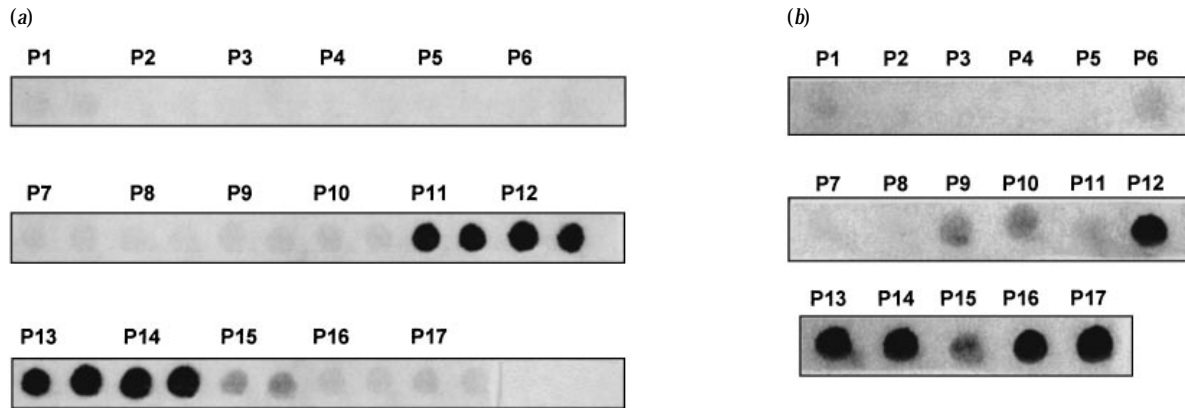


Fig. 4. Fine specificity of the anti-397–420 antibody response in BALB/c (a) and C57BL/6 (b) mice. An ELISA SPOTs Kit was employed using overlapping (by one residue) 8-mer peptides synthesized on a derivatized membrane to identify the location of the B-cell epitope(s). Each peptide was tested in duplicate for BALB/c mice. Pooled serum samples from the final bleed were tested.

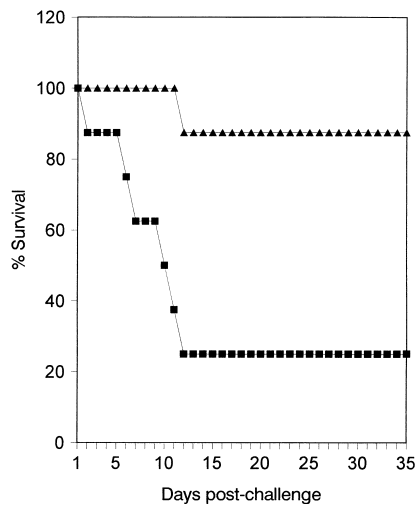


Fig. 5

Fig. 5. Percentage survival of C57BL/6 mice immunized i.p. with 397–420 peptide (▲) or PBS (■) against challenge with a neuroadapted strain of MV.

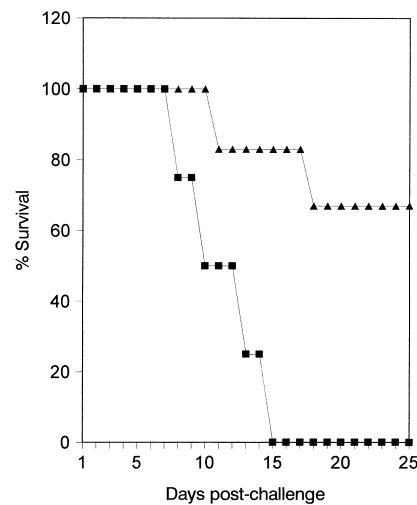


Fig. 6

Fig. 6. Percentage survival of BALB/c mice passively immunized with anti-397–420 antibodies (▲) or normal mouse serum (■) against challenge with a neuroadapted strain of MV.

However, although this chimeric peptide was immunogenic in six strains of mice, poor anti-404–414 responses were detected in C57BL/6 mice and no reactivity with MV was observed. In this report, peptide 397–420 was shown to induce strong anti-404–414 and anti-MV antibody responses in C57BL/6 mice. This suggests that the nature of the T-helper epitope might influence immune responsiveness.

When the fine specificity of the antibody response to the 397–420 peptide was characterized, using 8-mer peptides overlapping by one residue attached on a membrane, in both BALB/c and C57BL/6 mice, the specificity of the antibody response was directed towards the second half of the 397–420 sequence. C57BL/6 mice predominantly recognized the 408–420 region as compared to the 407–417 region

recognized by serum samples from BALB/c mice. This finding might reflect differences in relative topology between B- and T-helper epitopes in BALB/c and C57BL/6 mice. Although the topology of the T-helper epitopes has not been addressed in this study, it appears that the T-helper epitopes for BALB/c and C57BL/6 mice do not alter significantly the specificity of the antibody responses. This is supported by the finding that sera from both BALB/c and C57BL/6 strains of mice reacted with MV, thus suggesting that the 397–420 peptide adopts a conformation which mimics or resembles the arrangement of the B-cell epitope on the native F protein. Although the crystal structure of the protein has not yet been resolved, the predicted secondary structure of the F protein has three turns between residues 400–413 followed by an α -helical region (Richardson

et al., 1986). CD spectra analysis of the 397–420 peptide has shown its preference to adopt an α -helical conformation in an environment of low dielectric constant such as 50% TFE, which might resemble the environment at the cell surface. This indicates that prediction methods for the secondary structure of full-sized proteins might not be in agreement with the prediction of the conformation of short peptides in solution by CD. It could also be argued that although peptide 397–420 was shown to be linear (despite the presence of two cysteine residues), after *in vivo* immunization, cyclization might occur resulting in a more stable conformation which gives rise to an appropriate specificity antibody response. The later possibility is supported by the finding that peptide 398–419, where the two cysteine residues have been excluded, was weakly immunogenic in C57BL/6 mice (data not shown). Also studies by Francis *et al.* (1987) have demonstrated the importance of cysteine residues on the immunogenicity of synthetic peptides. Peptide 397–420 is located in a domain of the F protein, rich in cysteine residues (conserved among different members of the family *Paramyxoviridae*) (Richardson *et al.*, 1986). This suggests that these cysteine residues are important in contributing to the folding of the F protein into its native conformation via disulfide bridges.

Despite the lack of neutralizing activity of the anti-397–420 antibodies, protection against challenge with a neuroadapted strain of MV was shown to be afforded by active immunization with peptide in C57BL/6 mice or after passive transfer of anti-peptide antibodies in BALB/c mice. The inability of anti-397–420 antibodies to neutralize the virus *in vitro* despite its protective capacity *in vivo* suggests that the 397–420 region could possibly become more accessible on the surface of cells infected by the neuroadapted strain of MV as compared to the virus strain used for neutralization, where certain determinants such as 397–420 might be relatively inaccessible. This could facilitate the *in vivo* interaction of anti-397–420 antibodies with the F protein at the cell surface leading to the lysis of the infected cells in the presence of complement or interference with the fusion process (Portner *et al.*, 1987). Both *in vitro* neutralizing and non-neutralizing monoclonal antibodies have been shown to have the ability to prevent lethal viral infection in mice (Lefrancois, 1984; Love *et al.*, 1986).

In conclusion, these findings highlight the importance of the 397–420 region in protection in the MV encephalitis mouse model, and suggest that this epitope might be a good candidate for inclusion in a future MV synthetic peptide vaccine.

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