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Protection against measles virus encephalitis by monoclonal antibodies binding to a cystine loop domain of the H protein mimicked by peptides which are not recognized by maternal antibodies

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After immunization with measles virus (MV) several monoclonal antibodies (MAbs) were obtained, which reacted with peptides corresponding to the amino acids 361–410 of the haemagglutinin protein (MV-H). Three of these MAbs (BH6, BH21 and BH216) inhibited haemagglutination, neutralized MV *in vitro* and protected animals from a lethal challenge of rodent-adapted neurotropic MV. These MAbs reacted with the 15-mer peptides H381 and H386 defining their overlapping region 386–395 as a sequential neutralizing and protective epitope, which can be imitated by a short peptide. H381 and H386 share two Cys residues (C_{386}KGKIQALC$_{394}$ENPEWA) and for optimal MAb binding of peptide (or MV) disulphide bonds were required in addition to a linear C-terminal extension. Other MAbs bound to peptides C- (BH147, BH195) and N-terminally (BH168, BH171) adjacent to the loop but did not neutralize or protect. When sera from measles patients or from women of childbearing age were tested with the peptides corresponding to this haemagglutinating and neutralizing epitope (HNE), none of the sera recognized the 15-mer peptides of this region, while some reactivity was found to 30-mers homologous to different wild-type mutants. Its lack of recognition by maternal antibodies and its high degree of conservation would make the HNE loop an attractive candidate to include into a subunit vaccine, which could be administered during early childhood, independent of immune status.

Introduction

Current live attenuated measles virus (MV) vaccines have effectively reduced the morbidity and mortality of measles world-wide. However, while the incidence of measles has been reduced to sporadic cases in the northern hemisphere, the lack of resistance to passively transferred maternal antibodies and the thermal lability of the live measles vaccines are major impediments to their successful application in developing countries and to the global eradication of measles (Weiss, 1992). Although some progress has been made towards improved heat stability of live attenuated measles vaccines, subunit vaccines could potentially be distributed without the need for a cold chain. In addition, peptide-based vaccines could potentially be resistant to maternal antibodies, thus allowing early vaccination independent of immune status.

Maternal antibodies (Albrecht *et al.*, 1977) and hyperimmune globulin (Janeway, 1949) demonstrate the importance of antibodies in protecting exposed subjects from measles. MV haemagglutinin (MV-H) is known to be the prime target for neutralizing and protective antibodies (Giraudon & Wild, 1985; Varsanyi *et al.*, 1987).

Competitive binding assays with monoclonal antibodies (MAbs) have been used to identify distinct antigenic sites of the H protein (Sheshberadaran & Norrby, 1986; Carter *et al.*,

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1982; Giraudon & Wild, 1985; ter Meulen *et al.*, 1981). Hu *et al.* (1993) and Liebert *et al.* (1994) have mapped neutralizing epitopes of the MV-H by sequencing escape mutants resistant to neutralizing antibodies. Thus, amino acids were identified that are important for the integrity of the MAb binding sites. Neutralizing antibodies are mostly directed against conformational epitopes. However, linear epitopes have also been found to be neutralizing and sometimes protective e.g. in foot-and-mouth disease virus (Francis *et al.*, 1991), influenza virus (Shapira *et al.*, 1984), polio virus (Emini *et al.*, 1983) and human immunodeficiency virus (Wang *et al.*, 1991). In morbilliviruses, the potential of peptides for immunization has been demonstrated with a neutralizing and protective epitope of the MV fusion protein (Obeid *et al.*, 1995; Steward *et al.*, 1995).

Linear binding sites of MV-H-specific antibodies have been mapped with sera from late convalescent donors by peptide ELISA using a full set of overlapping peptides of the MV-H protein (Muller *et al.*, 1993). Using peptide 185–195 of the MV-H, a rabbit polyclonal serum was obtained which neutralized *in vitro* (Mäkelä *et al.*, 1989).

In the present study, we have developed MAbs that defined a cystine loop protruding at the MV-H surface that represents a neutralizing and protective epitope that can be mimicked by a short linear peptide and that is not recognized by human maternal antibodies.

Methods

■ **Reagents.** The MV Edmonston strain (ATCC VR-24) was grown in Vero cell cultures and its early passage supernatants were used for neutralization assays. Haemagglutination-inhibition (HAI) assays and MV ELISA were performed with MV concentrated by ultrafiltration. For Western blot and for one immunization (BH216) the virus was further concentrated and purified by sucrose-gradient centrifugation (Muller *et al.*, 1995). Virus used for all other immunizations was purified by affinity chromatography and inactivated with β-propiolactone. Ltk^- cells transfected with H (Ltk^-H; kindly provided by F. Wild and P. Beauverger; Beauverger *et al.*, 1993, 1994) and a persistently infected human Epstein–Barr virus (EBV) -transformed B cell line (WMPTT; gift from B. M. Chain, University College Medical School, London, UK) were used to test the reactivity of the MAbs with MV or MV-H protein by flow cytometry as described (Muller *et al.*, 1995).

■ **Production of MAbs.** 8–10-week-old BALB/c mice were immunized by intraperitoneal or multifocal subcutaneous injections with purified native or denatured (5 min boiling in 1 mg/ml SDS under non-reducing conditions) MV emulsified in incomplete Freund's adjuvant (Sigma). The animals were boosted with the same antigen preparation on day 21 and 38 (denatured MV) or day 49 (native MV). On day 41 or 52, spleen cells were explanted and fused with Sp2/0 myeloma cells to generate MAbs. Hybridomas specific for MV by ELISA or by Western blot were cloned by limiting dilution in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 mM-hypoxanthine, 1 mM-glutamine, 1 mM-sodium pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS) (all from Gibco). Antibodies were purified from the same but FBS-free medium or from ascites and purified by affinity chromatography on a protein G column (Pharmacia). Concentrations were estimated by A_{280} determination. Ig subclasses and isotypes were determined with the mouse MAb isotyping kit (Hycult Biotechnology)

■ **Peptides.** 121 15-mers overlapping by 10 amino acids and covering the whole sequence of the MV-H protein (Edmonston strain; Alkhatib & Briedis, 1986) and truncation, elongation and substitution analogues of peptide H381 and H386 were synthesized by simultaneous multiple peptide synthesis (Wiesmüller *et al.*, 1992). The peptides were modified at the C terminus by two ε-amino caproic acid residues and one lysine residue which separate the peptide from the biotin residue which was coupled to the $N\varepsilon$-amino group of lysine amide. In Fig. 4 peptides were biotinylated at their N terminus using a similar spacer. All peptides are designated by the position of their N-terminal amino acid. Peptides were reduced with 140 mM-2-mercaptoethanol. Even much higher concentrations did not directly interfere with the ELISA assay, as was measured with an antibody specific for a peptide containing a single Cys. During the different ELISA steps, reduced peptides were protected from oxidation with 0·01% vitamin C. Peptides were oxidized by bubbling air through a 5 µg/ml solution.

■ **ELISA.** The ELISA based on the biotinylated peptides was described previously (Fournier *et al.*, 1996). In brief, microtitre plates (Maxisorp; NUNC) were coated with 20 µg/ml highly purified streptavidin (a gift from L. Seik, Mediagnost, Tübingen, Germany). Biotinylated peptides (5 µg/ml) were immobilized by incubation for at least 1 h at room temperature. Binding of the biotinylated peptides to the plate was verified by competition with biotinylated alkaline phosphatase (Vector Laboratories). Free binding sites were saturated with 1% BSA (Sigma) in TBS. In some assays with MAbs the blocking buffer contained 10% BSA, 10% saccharose and 2% v/v normal goat serum (ICN) in PBS. The plates were either incubated for 1·5 h at 37 °C or overnight at 4 °C with mouse sera or hybridoma supernatants diluted 1:200- or 1:20-fold, respectively, in dilution buffer (10 mM-TBS, 1% BSA, 0·1% Tween, pH 7·4).

The ELISAs were developed with alkaline phosphatase-conjugated goat immunoglobulin (whole molecule) specific for the γ-chain of mouse or human IgG (Sigma) at a dilution of 1:500 in dilution buffer using *p*-nitrophenylphosphate (Sigma) as a substrate. Absorbance was measured at 405 nm.

■ ***In vivo* challenge/protection experiments.** 13–16-day-old, specific-pathogen-free (SPF) BALB/c mice were treated with a single intraperitoneal injection of purified MAbs (56–364 µg). 1 day later they were challenged by intracerebral injection of 12500 $TCID_{50}$ of BALB/c-adapted CAM MV strain derived from the CAM/RB strain (a gift from U. G. Liebert, Würzburg, Germany; Liebert & ter Meulen, 1987).

■ **Human sera.** Blood was drawn after informed consent from 320 consecutive out-patients visiting the Laboratoire National de Santé (Luxembourg) for a diagnostic venous puncture. Neutralization (NT) and HAI titres were performed as described earlier (Norrby & Gollmar, 1972) with some minor modifications (Muller *et al.*, 1995). Complement-fixing (CF) titres of human sera were determined with a commercial kit (Virion, CH-6330 Cham). Mean $\log_2$ titres were $7{\cdot}7 \pm 1{\cdot}2$, $7{\cdot}2 \pm 1{\cdot}8$, $4{\cdot}4 \pm 1{\cdot}9$ for NT, HAI and CF, respectively. Twenty sera from women of child-bearing age with high NT and HAI titres were tested (Table 2) with different peptides corresponding to the epitope described here. Donors were selected irrespective of their vaccination status. Five additional sera with elevated MV titres were obtained from a recent measles outbreak in Taiwan (Lee *et al.*, 1992, 1995).

Results

Epitope mapping with MV-H peptides

A panel of 60 H-specific MAb clones was obtained after

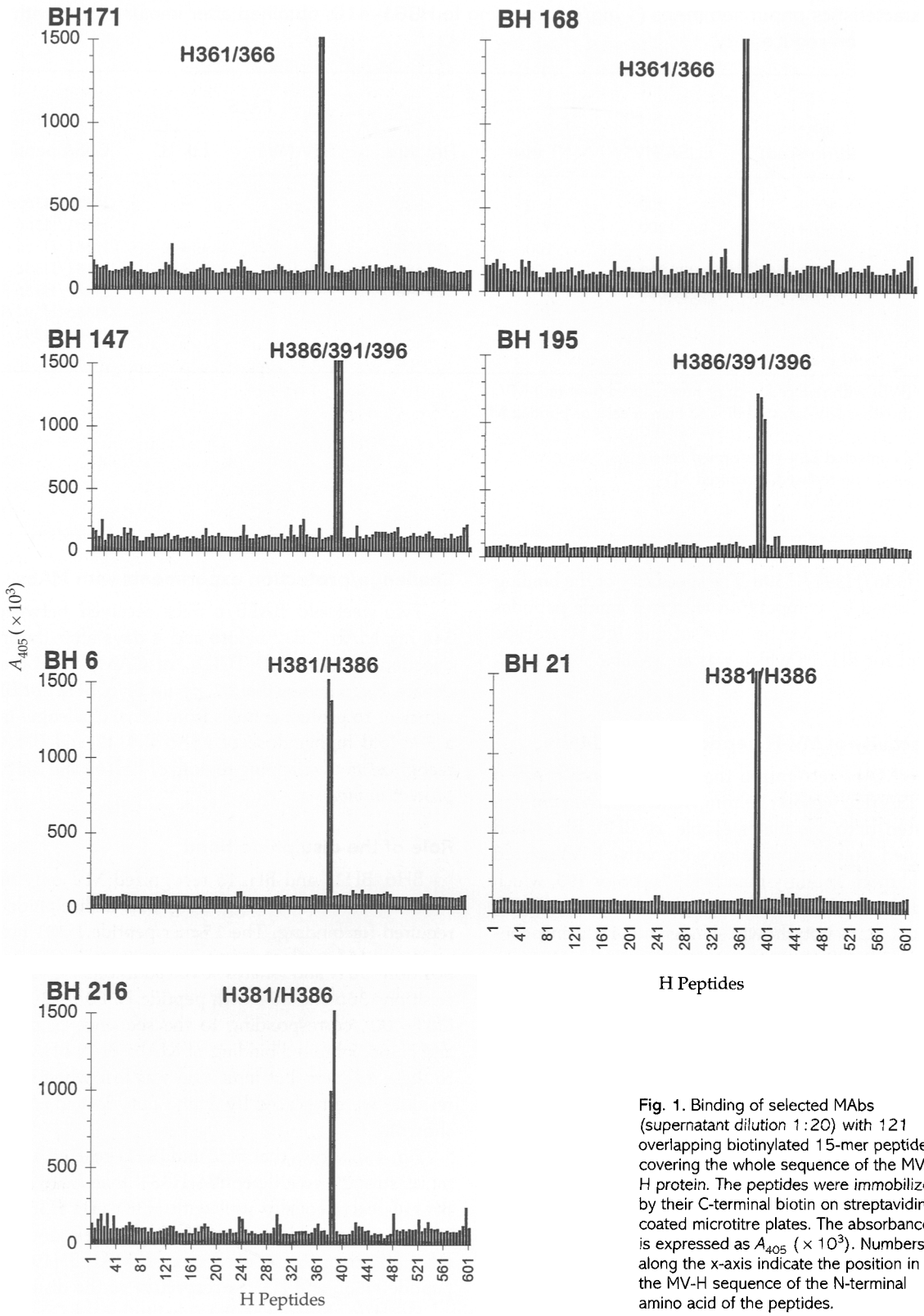

Fig. 1. Binding of selected MAbs (supernatant dilution 1:20) with 121 overlapping biotinylated 15-mer peptides covering the whole sequence of the MV-H protein. The peptides were immobilized by their C-terminal biotin on streptavidin-coated microtitre plates. The absorbance is expressed as A_{405} ($\times 10^3$). Numbers along the x-axis indicate the position in the MV-H sequence of the N-terminal amino acid of the peptides.

immunization with native or denatured MV. Linear epitopes of these clones were mapped using an ELISA based on biotinylated peptides. Seven MAbs were found to react with peptides corresponding to the amino acids 361–410 (Fig. 1). MAbs BH168 and BH171 reacted with peptides H361/H366; BH6, BH21 and BH216 with H381/H386; and BH147 and

Table 1. Characteristics of purified MAbs (1 mg/ml) mapping to H361–410, obtained after immunization with native MV or denatured non-reduced MV

MAbs	WB* (red/non-red)	ELISA MV†	NT titre	HAI titre	FACS EBV-MV§	FACS Ltk⁻H	ELISA peptides‡
BH171‖	+/+	200	< 2	< 20	−	−	H361/H366
BH168‖	+/+	200	< 2	< 20	−	−	H361/H366
BH6¶	−/+	200000	100	20480	+++	+++	H381/H386
BH21¶	−/+	200000	100	20480	+++	+++	H381/H386
BH216¶	−/+	200000	100	20480	+++	+++	H381/H386
BH147‖	+/+	100	< 2	< 20	−	−	H386/H391/H396
BH195‖	+/+	100	< 2	< 20	−	−	H386/H391/H396

* Western blot (WB) with reduced (red) or non-reduced (non-red) MV.
† Reactivity with either MV-infected diploid human cells or purified MV.
‡ See Fig. 1.
§ Persistently MV-infected EBV-transformed cells.
‖ Generated against non-reduced denatured MV.
¶ Generated against native MV.

BH195 with H386/H391/H396. The specificity of the binding has been confirmed by competition with free amide peptides (data not shown). The MAbs were of the IgG1κ isotype subclass except for BH216 which was an IgG2bκ.

Functional activity of MV-H peptide-specific MAbs

The above MAbs recognized the MV both in a certified diagnostic ELISA (Enzygnost; Behringwerke) or when purified MV was coated onto the plates (Table 1). BH6, BH21 and BH216 (obtained after immunization with native MV) reacted about 500-fold more strongly than BH171, 168 or 195, which were derived from animals immunized with (non-reduced) denatured virus (data not shown). Under non-reducing conditions they all identified a 160 kDa protein in the Western blot, corresponding to the dimeric form of the H protein (Gerlier *et al.*, 1988; Rima, 1983). This was confirmed both with purified MV and recombinant H protein. Under reducing conditions, BH168, BH171, BH147 and BH195 reacted with an 80 kDa protein, while the reactivity of BH6, BH21 and BH216 with the MV was lost. MAbs BH6, BH21 and BH216 recognized H on the surface of the MV-infected EBV-transformed cell line and the H-transfected Ltk$^-$ cells. BH171, BH168, BH147 and BH195 were essentially negative on these cells. (Fig. 2). Recombinant H protein inhibited binding of BH21, BH6 and BH216 to peptide H386, demonstrating that the same antibodies react with both antigens. Only BH6, BH21 and BH216 neutralized MV *in vitro* and inhibited haemagglutination of monkey erythrocytes (Table 1). Although the binding sites of these peptides partially overlapped (amino acids 386–400) with those of BH147 and BH195, the latter did not display any functional activities.

Challenge/protection experiments with MAbs

Two-week-old BALB/c mice received between 65 and 344 mg MAb 1 day before and 4 days after the intracranial injection of $1{\cdot}25 \times 10^4$ $TCID_{50}$ of CAM/BALB brain homogenate. Fig. 3 shows that 62 ± 6 µg BH6, BH21 or BH216 was sufficient to protect animals from lethal challenge. In contrast, a 5–6-fold higher dose of MAb BH147 and BH195 (which recognize an overlapping region) or BH168 and BH171 did not protect *in vivo*.

Role of the disulphide bond

BH6, BH21 and BH216 recognized MV only under non-reducing conditions, suggesting that a disulphide bridge is required for binding. The 15-mer peptide H381 has a Cys in position 381 and shares two additional Cys residues at positions 386 and 394 with peptide H386. A 20-mer peptide (381–400), corresponding to the sequence of peptide H381 and H386, inhibited binding of MAbs BH6, BH21 and BH216 to these 15-mers, but inhibition was lost when the three Cys residues were replaced by amino butyric acid (Abu; data not shown).

Fig. 4(*a*) shows that BH6 and BH21 reacted about fourfold more strongly with peptide H381 than with H386, while BH216 reacts equally well with H381 and H386. The three MAbs recognized the oxidized peptides 4–64-fold more efficiently than the reduced species. MAb BH195 reacts with peptide H386 and H391 irrespective of the disulphide bond.

Fig. 4(*b*) shows that the substitution of Cys-386 by Abu decreases binding of MAbs BH6 and BH21 to oxidized H386 almost 100-fold. Substitution of Cys-394, either alone or together with Cys-386 completely blocks binding of these MAbs. Binding of BH216 was considerably less sensitive to

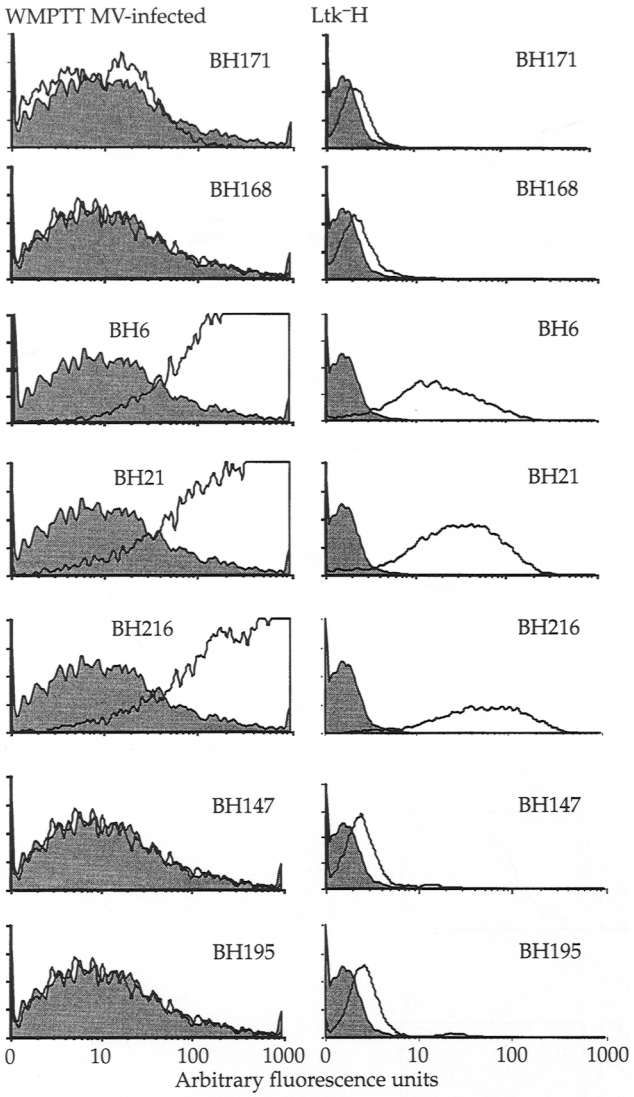

Fig. 2. Binding of MAbs to MV-infected EBV-transformed human B cells (WMPTT; left panels) and Ltk⁻H cells (right panels) measured by flow cytometry. Background binding corresponds to the FITC-conjugate alone on EBV-MV cells (GaM) or Ltk⁻H cells. The difference in background between the WMPT and the Ltk⁻H cells is mainly due to differences in autofluorescence and to a lesser extend to higher background binding of the FITC-conjugate. Background binding of MAbs to the uninfected EBV cells and to Ltk⁻ wild-type cells was similar to the background of GaM on the corresponding H expressing cells (data not shown).

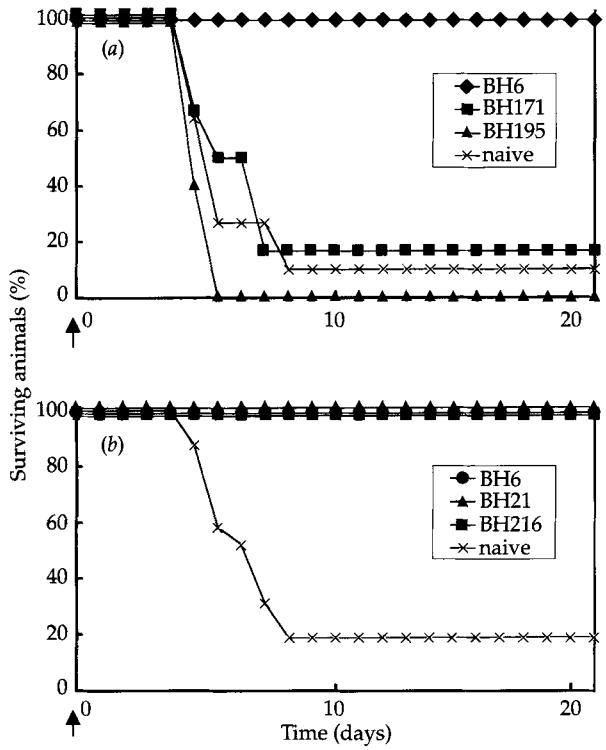

Fig. 3. Challenge/protection experiment with mice passively immunized on day −1 with 315 μg BH6, 344 μg BH171, 330 μg BH195 (*a*) or 62 ± 6 μg BH6, BH21 or BH216 (*b*). On day 0, mice were challenged with an intracranial injection of CAM mouse-adapted MV. Naive mice were challenged without prior antibody transfer. Each group represents 6–8 mice from two independent experiments.

reduction and to Abu substitution. Virtually no difference in binding was found between oxidized and reduced Abu-derivatives. The reaction of BH195 is independent of Abu substitution or the oxidation state. The results showed that the cystine loop is required for optimal binding of the neutralizing antibodies BH6, BH21 and to a lesser extent BH216. However, it also shows that some of the reactivity is to a linear part of the peptide. Therefore, peptide H386 was truncated from the C terminus.

C-terminal truncation of peptide H386 showed that both in the reduced or oxidized state the 14-mer is required for maximal binding of MAbs BH6, BH21 and BH216 (Fig. 4*c*). The antibodies did not react with the 10-mer or shorter peptides even at the highest concentrations used (not shown). C-terminal elongation did not further enhance binding. Thus, in addition to the S–S loop, the C-terminal linear tail contributes to the binding affinity. It is therefore not surprising that peptide H383–397, which lacked both, did not react with anti-MV-serum (Obeid *et al.*, 1994).

Sequence alignment

The sequence H381–405 of different MV isolates is fully conserved in all vaccine strains (Fig. 5). Limited mutations have been found in recent wild-type isolates. Most mutations were found in the IP3 strain, a subacute-sclerosing panencephalitis (SSPE) isolate from the early 1970s, which was shown to have a markedly reduced haemadsorption activity (Cattaneo *et al.*, 1989; Burnstein *et al.*, 1974). Escape mutants have been described in this region by Hu *et al.* (1993) and Liebert *et al.* (1994). The H protein of the MV shares about 60% identity to rinderpest virus H (Tsukiyama *et al.*, 1987; Yamanaka *et al.*, 1988) and about 34% with canine distemper virus H (Curran *et al.*, 1991). The amino acid sequence 381–400 of the H protein of different morbilliviruses (Fig. 5) shows very little identity

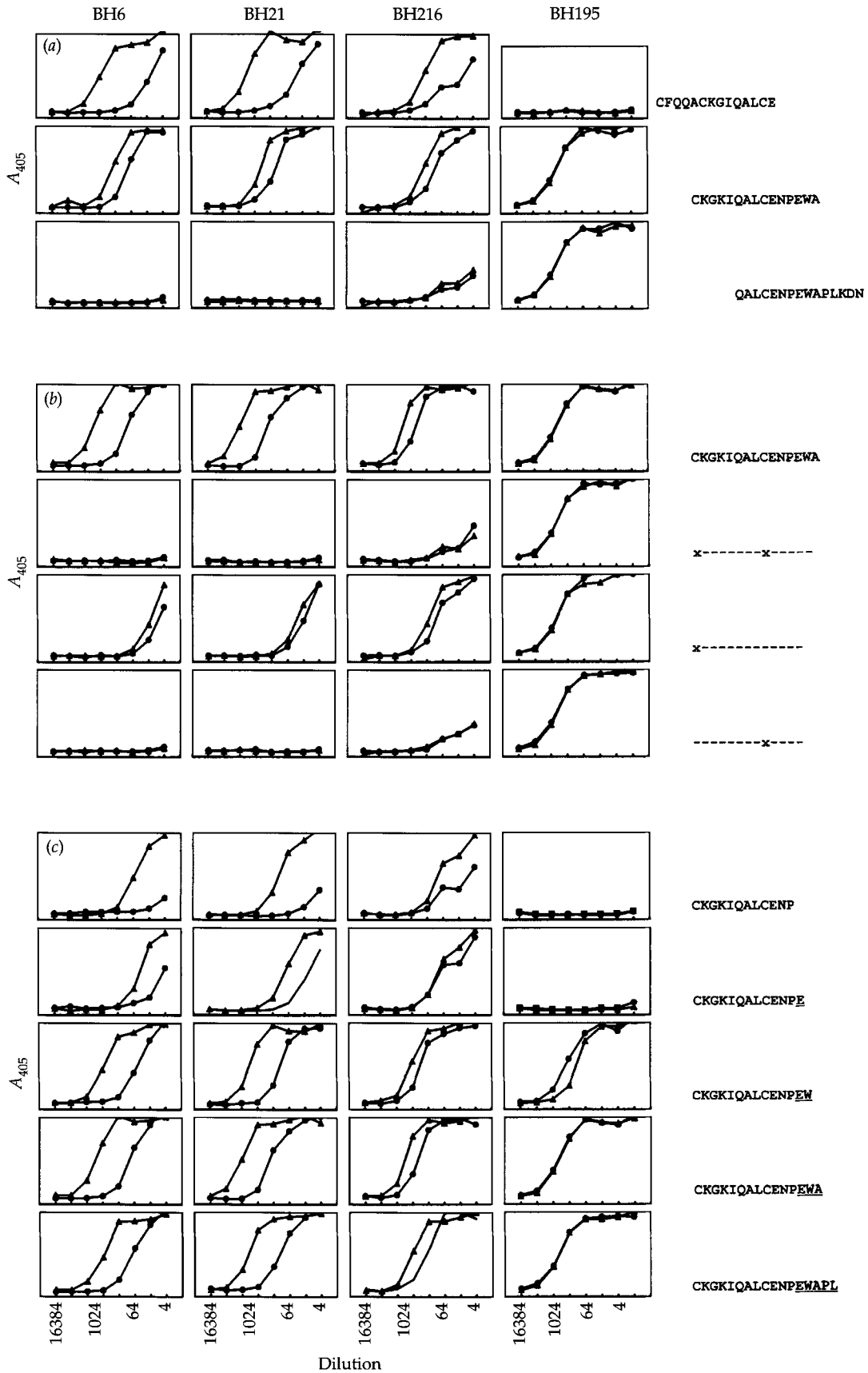

Fig. 4. For legend see opposite.

NAMES	SEQUENCES	REFERENCES
	<-----H381----->	
	<-----H386---->	
	<-----H391---->	
EDWT*	CFQQACKGKIQALCENPEWAPLKDN	Alkhatib and Briedis, 1986
PAMMEAHA*	----------CV-----	Gerald et al., 1986
MEAHAA*	---------N----------	Rota et al., 1992
MEAHAAA*	--------R----------	Rota et al., 1992
MEAHAAD*	---------N----------	Rota et al., 1992
MVHGRNAA*	----------S	Hu et al., 1993
MV08411*	---H----------	Hummel et al., 1994
MV08414*	---H----------	Hummel et al., 1994
S73869*	---------N----------	Saito et al., 1994
IP-3-Ca	------R-EV-----D--------G	Cattaneo et al., 1989
A	----------	Cattaneo et al., 1989
B	-------N----------H	Cattaneo et al., 1989
MIBE	----------G	Cattaneo et al., 1989
SH	----------	Schulz et al., 1992
TT	----------	Schulz et al., 1992
CL	----------	Schulz et al., 1992
SE	----------	Schulz et al., 1992
AIK-c	----------	Mori et al., 1993
Chi-1	---------N----------	Rota et al., 1994
SanD	---------N----------	Rota et al., 1994
Chi-2	--------R----------	Rota et al., 1994
Mcl	----------	Rota et al., 1994
JM	----------	Rota et al., 1994
EWB	----------	Rota et al., 1994
CAM	----------	Rota et al., 1994
MOR	----------	Rota et al., 1994
ZAG	----------	Rota et al., 1994
YA	----------	Komase et al., 1990
Lec-WI	------D----------	Hu et al., 1993
CAM/NC32	------S----------	Liebert et al., 1994
CAM/K71	----------K----------	Liebert et al., 1994
RIND L	-RRE--RE-PPPF-NSTD-E--EAG	Tsukiyama et al., 1987
RIND K	-RLE--RFRPPPF-NSTD-E--EAG	Yamanaka et al., 1988
CDV	-LES--QR-TYPM-NQAS-E-FGGR	Curran et al., 1991

*: EMBL references

Fig. 5. Alignment of the haemagglutinin-neutralizing epitope (HNE) region (amino acids 381–405 of MV-H) of different MV isolates (EDWT to YA), of *in vitro* induced antibody escape mutants (Lec-Wi to CAM/K71) and of other morbilliviruses (rinderpest virus L to canine distemper virus). Only amino acids differing from the Edmonston wild-type strain (EDWT; Alkhatib & Briedis, 1986) are shown. The peptides used in this study are indicated above the alignments. We thank B. Rima (Belfast, UK) for making available parts of his data bank.

but the Cys residues are fully conserved, indicating that the local loop formed by disulphide bound is functionally critical.

Reactivity of human sera with HNE peptides

To study whether maternal antibodies could react with this neutralizing epitope, a panel of sera obtained from women of child-bearing age (Table 2) was tested against peptides H381 and H386 and against 30-mer peptides corresponding to most of the mutations shown in Fig. 6. The sera were selected solely on the basis of high NT and HAI titres (see Methods). Essentially, three types of reactions were found (Fig. 6): three sera reacted strongly with the same 9 of the 11 30-mers, 10 gave a weak reaction and 7 sera did not react with any peptide. One of the two peptides that were not recognized by any serum contained an additional Cys in position 401. No reactivity was found against the 15-mers H381, H386 or H391. We also tested sera collected at a recent MV outbreak in Taiwan (S13, S22, S39, S41, S42; Lee *et al.*, 1992; Table 2). One serum reacted with a single 30-mer and not with any of the 15-mer peptides of the neutralizing epitope described here (Fig. 6).

Discussion

After immunization with native or denatured MV, several MAbs were obtained that reacted with peptides corresponding to the sequence H361–410 (Table 1). Three MAbs (BH6, BH21 and BH216) inhibited virus-induced haemagglutination of monkey erythrocytes, neutralized the virus *in vitro*, and protected animals from a lethal challenge of rodent-adapted neurotropic MV. These three MAbs reacted with the 15-mer peptides H381 and H386 defining H381–400 as a linear neutralizing and protective epitope. These peptides correspond to a small cysteine cluster region (Cys-381, Cys-386 and Cys-394). In Western blots, BH6, BH21 and BH216 reacted with MV only under non-reducing conditions. For optimal binding, peptide H381 and H386 had to be oxidized, suggesting that a disulphide bond is required, which is formed by both peptides. Since these peptides share Cys-386 and Cys-394, but not Cys-381, a loop formed by the former two residues is the likely target structure for the neutralizing antibodies. This interpretation is also supported by binding studies with substitution analogues in which the -SH group of the Cys-386 and/or Cys-394 is replaced by -OH. It is therefore somewhat surprising that H381 binds MAbs BH6 and BH21 fourfold better than H386. Data from site-directed mutagenesis of individual Cys residues are more compatible with a loop structure formed by Cys-381 and Cys-394 (Hu & Norrby, 1994). It is therefore possible that differences in binding affinity to the overlapping region 386–395 of peptides H381 and H386 only reflect the more or less tight curvature of the Cys loop in these two peptides.

Studies with the Abu analogues and the C-terminal truncations showed that there was also a low-affinity binding component to the linear C-terminal tail of H386. Recognition of amino acids of the C-terminal tail [mainly (E)WA-COOH] by BH6, BH21 and BH216 does not require oxidation.

Fig. 4. Titration of MAb BH6, BH21, BH216 and BH195 on biotinylated peptide analogues of H386 immobilized to streptavidin-coated plates. The concentrations of MAbs corresponding to the 1:2 dilutions were: 10 μg/ml BH6; 3·5 μg/ml BH21; 11·2 μg/ml BH216; 2·6 μg/ml BH195. (*a*) Reactivity of these MAbs with peptide H386 (15-mer) and adjacent 15-mers H381 and H391; (*b*) shows the reaction with amino-butyric acid (X) single and double substitution analogues of H386: H386(Cys-386/394 → Abu), H386(Cys-386 → Abu), H386(Cys-394 → Abu); (*c*) displays binding to C-terminal truncation and elongation analogues of H386. ▲, oxidized peptides; ●, reduced peptides. The data is shown as A_{405} on the y-axis, the scale ranging for all panels from 0 to 2·0. The X-axis shows the dilutions ranging in fourfold dilution steps from undiluted to 1:16384 of the above stock solutions.

Table 2. Characteristics of sera from women of child-bearing age (samples HS1005–HS1163) and from measles patients from an outbreak in Taiwan (samples S13–S51)

Sample	Age (years)	MV exposure*	HAI†	NT†	CF†	HNE-ELISA‡
HS1005	39	?	8·0	9·0	7	+
HS1006	31	?	8·5	8·5	5	+
HS1010	38	D (7)	10·0	11·0	6	+
HS1013	29	D (10)	10·0	11·0	6	+ + +
HS1018	27	?	9·5	8·5	6	+
HS1024	27	?	8·0	8·5	5	+
HS1031	23	?	8·5	6·0	3	+
HS1036	34	?	8·0	7·5	4	−
HS1039	31	D (3–8)	10·0	10·0	8	+ + +
HS1118	25	D (5–10)	8·0	8·5	7	−
HS1122	36	D (9)	9·0	9·0	7	−
HS1135	33	D (11)	8·5	8·5	6	−
HS1144	46	?	9·0	9·0	NT	+
HS1151	31	D (8–9)	9·0	9·0	7	+
HS1180	26	D (6–7)	8·5	7·5	6	−
HS1196	29	D (12)	8·5	8·0	5	−
HS1199	25	?	8·0	8·0	4	−
HS1204	26	?	8·0	8·5	6	+
HS1245	32	D (3–5)	8·5	NT	5	+
HS1163	45	?	10·5	8·5	7	+ + +
Mean	±SD		8·3 ± 2·0	8·6 ± 1·2	5·7 ± 1·3	
S39	8	D[12]	8·6	0	6	
S41	10	D[8]	9·2	7·3	6	
S42	2	D[22]	7·3	6·9	10	
S45§	42	D[1]	8·6	6·3	3	
S51§	42	D[3]	8·6	6·9	3	

* D( ) = disease at the age of ( ); D[ ] = disease, serum drawn [days after rash].
† Log_2 titre.
‡ + + +, strong; +, weak; −, negative reaction with 30-mer peptides as shown in Fig. 6.
§ Serum of the same donor.

Two additional MAbs, BH147 and BH195, mapped to the sequence H386–410 partially overlapping with the peptides recognized by the above neutralizing MAbs. In contrast to the neutralizing antibodies, BH195 was insensitive to Abu substitution and to reduction, demonstrating that these MAbs did not require the loop structure for binding. C-terminal truncation analogues demonstrated that Trp-399 was critical for binding of BH195. The comparison of BH195/BH147 which did not neutralize, with BH6/BH21/BH216 highlights the importance of the Cys loop for neutralization.

Two MAbs (BH171 and BH168) reacted with a region located N-terminal of the loop. This region corresponds to the most hydrophilic region of the protein and neutralizing MAbs have induced escape mutants with mutations in this region (377R → Q, 378M → K; Liebert *et al.*, 1994). Our antibodies, however, did not neutralize the virus, inhibit haemagglutination or protect *in vivo*. Mäkelä *et al.* (1989) have also found reactivity to this region in an anti-MV polyclonal serum, but anti-peptide H368–377 antibodies did not display any functional activity, although they reacted with MV. These results suggest that this region may contain a neutralizing epitope that is also not readily mimicked by the corresponding linear peptide.

Our antibodies against the H381–400 region define a local loop protruding from the surface of the MV-H that contains an epitope involved in haemagglutination and neutralization ('HNE loop') and which can be imitated by short peptide loops. Such peptides are of potential interest for incorporation into a subunit vaccine. Proteins and peptides usually induce antibodies of the IgG1 subtype, which lacks efficiency for complement fixation and FcγRI binding of the IgG2a isotype (Neuberger & Rajewsky, 1981; Ravetch & Kinet, 1991), the most important effector antibody in viral infections (Ishizaka *et al.*, 1995). Our *in vivo* challenge experiments demonstrated that protection via MAbs targeted to the HNE loop is independent of these effector functions, since both IgG1 (BH6 and BH21) and IgG2b (BH216) isotypes were protective.

In human immunodeficiency virus (HIV), such a loop (V3)

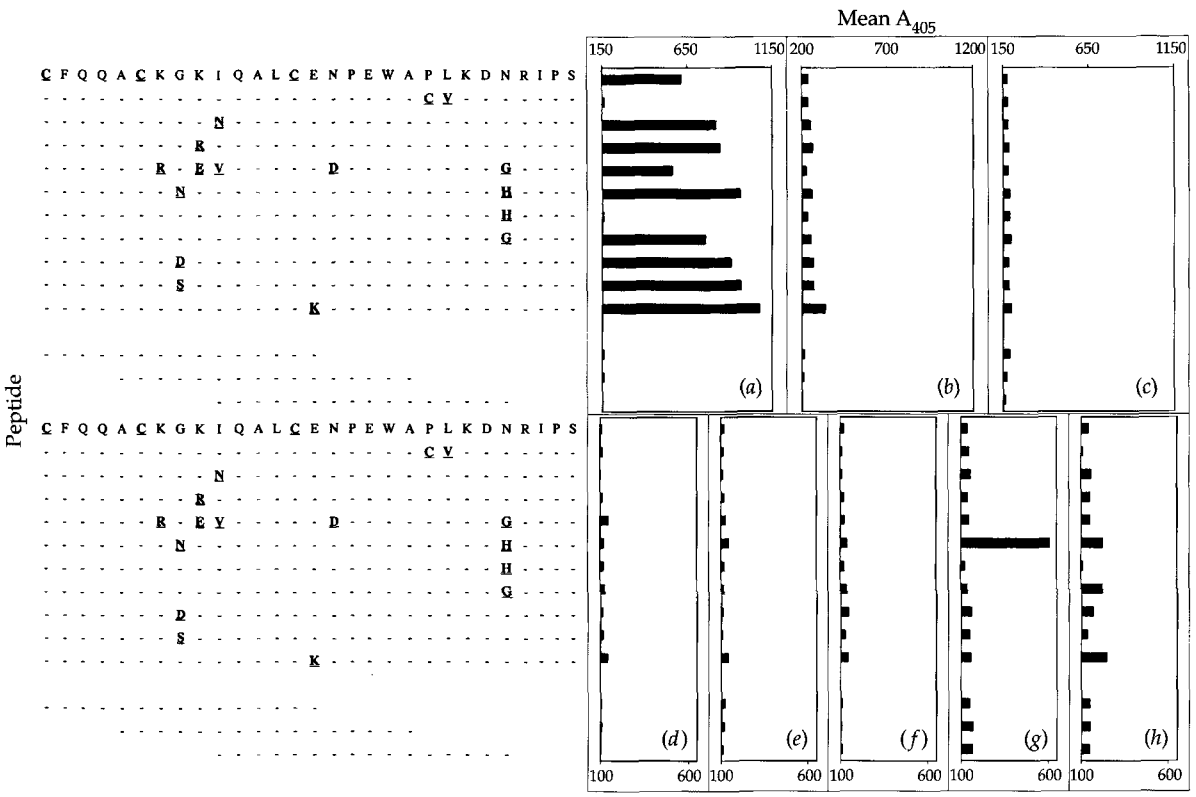

Fig. 6. Reactivity of human sera (Table 2) with the 15-mers H381, H386 and H391 and with different 30-mer peptides of the H381–410 region homologous with virus isolates of Fig. 5. The upper panels show three examples of sera from women of child-bearing age with strong (HS1155), weak (HS1144) and negative (HS1122) reaction with the 30-mers. The strong (+ + +), weak (+) and negative (−) reaction of the other women's sera are shown in Table 2. The lower panel shows five sera from a measles outbreak in Taiwan (same order as in Table 2). Data represent mean A_{405} ($\times 10^3$) of a biotinylated peptide ELISA. Backgrounds [100–200 × ($A_{405} \times 10^3$)] defined as the reactivity of non-reacting peptides were truncated.

has emerged as the principal neutralizing linear determinant in the envelope protein (Wang *et al.*, 1991; Ahlers *et al.*, 1993). Peptides mimicking this structure have generated neutralizing and protective antibodies in chimpanzees (Fultz *et al.*, 1992; Goudsmit *et al.*, 1988). Clinical studies are underway to investigate the potential of the V3 loop as a human vaccine (Walker & Fast, 1994). However, the V3 loop as a target for protective antibodies suffers from its extensive sequence variability (Javaherian *et al.*, 1989; Goudsmit *et al.*, 1991). In contrast, only limited numbers of mutations of the HNE loop of MV have been reported (Fig. 5) suggesting that extensive variability in this region cannot be tolerated. Although the region bears little identity to other morbilliviruses such as rinderpest virus and canine distemper virus, the conservation of the cysteines suggests that the HNE loop is essential and will not easily disappear under the selective pressure of antibodies.

In this context, observations in the rodent-adapted CAM strain which have associated this region with neurovirulence are of interest (Liebert *et al.*, 1994). While an E-395 → K mutation removed neurovirulence, the G-388 → S mutation lowered neuropathology (Liebert *et al.*, 1994). The IP3 wild-type isolate derived from an SSPE patient also showed mutations in this region (Fig. 5; Cattaneo *et al.*, 1989; Burnstein *et al.*, 1974). Further studies with mutants will be required to test whether antibodies to this region may also potentiate disease.

One of the benefits of peptide-based MV vaccines is their potential to escape maternal antibodies transferred from mother to the child. Such a subunit vaccine could conceivably be used to induce neutralizing antibodies in the presence of antibodies from vaccinated or wild-type MV-infected mothers. We showed (Muller *et al.*, 1993) that sera from late convalescent donors did not react with 15-mers corresponding to the HNE loop of the Edmonston strain. It is conceivable that antibodies were missed which were induced by wild-type MV with non-cross-reactive mutations in the HNE loop. To minimize this possibility in the present study, high titre sera from women of child-bearing age and from patients recovering from measles (Lee *et al.*, 1992, 1995) were reacted here with a set of 30-mer peptides corresponding to most known mutations in this region. Few sera reacted strongly with the longer peptides but none reacted with the 15-mers which fully represented the neutralizing and protective epitope. A number of isolates from a single outbreak showed no mutations in this region, confirming that the HNE loop is not under a strong

selective pressure from neutralizing antibodies (Outlaw & Pringle, 1995). This suggests that HNE loop peptides could potentially be suitable for immunization in the presence of neutralizing MV antibodies. Further studies will be required, however, to investigate cross-reactivity of HNE loop-specific antibodies of isolates obtained from different geographical areas.

Note added in proof. Cystine loops have recently been described in a number of proteins as a 'noose' motif which provides high surface accessibility to the amino acids contained in the loop (Lapthorn *et al.*, *Nature Structural Biology* **2**, 266–268). The HNE loop described here would therefore represent a Haemagglutinin Noose Epitope.

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