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Antibodies to a new linear site at the topographical or functional interface between the haemagglutinin and fusion proteins protect against measles encephalitis

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The haemagglutinin protein (H) of measles virus (MV) binds to susceptible cells and collaborates with the fusion protein (F) to mediate fusion of the virus with the cell membrane. Binding and fusion activity of the virus can be monitored by haemagglutination and haemolysis, respectively, of monkey erythrocytes. Most monoclonal antibodies (MAbs) with haemolysis inhibiting activity (HLI) are either MV-F specific and do not inhibit haemagglutination (HI), or they bind to MV-H and are HI by interfering with virus binding. We describe here a small panel of H-specific MAbs (BH47, BH59, BH103, BH129) which bind to a new linear neutralizing epitope, H244–250 (SELSQLS; NE domain), and which prevent virus–cell fusion (HLI) but not virus binding (HI). These antibodies also protect against MV encephalitis in an animal model. They do not compete with an HLI /HI antibody (BH216) which binds to the haemagglutinin noose epitope (HNE). The antibodies described here and the HNE-specific antibodies are functionally distinct and define two topographically non-overlapping interfaces, supposedly with a bias towards the host cell MV-receptor and the fusion protein respectively. The proximity of the CD46 downregulating amino acid Arg-243 may suggest a functional link between the domain described here and the CD46 binding domain. This new protective linear site is also of potential interest for the design of a subunit-based vaccine.

Introduction

Despite efficient vaccines, measles continues to cause considerable morbidity and mortality worldwide. Improved vaccination strategies may well be required to achieve the World Health Organization goal of measles eradication (Nokes & Cutts, 1993). Current live-attenuated vaccines require expensive cold-chains and lack resistance to transplacentally acquired maternal antibodies. Subunit vaccines based on peptides would be stable under tropical conditions and would potentially be resistant to maternal antibodies (Albrecht *et al.*, 1977; Obeid & Steward, 1994). The potential of peptides for immunizing against morbilliviruses has been demonstrated by M. W. Steward and colleagues (Obeid *et al.*, 1995). Although the fusion protein can be the target of neutralizing antibodies (Malvoisin & Wild, 1990), most of the protective activity of measles virus (MV) antibodies is directed against the haemagglutinin protein (MV-H; Giraudon & Wild, 1985). Therefore, protective B cell epitopes of MV-H would be important components of such a subunit vaccine. Various strategies have been used to map antigenic sites of the MV-H protein (Sheshberadaran & Norrby, 1986; Carter *et al.*, 1982; Hu *et al.*, 1993; Liebert *et al.*, 1994; Hummel & Bellini, 1995; Mäkelä *et al.*, 1987; Obeid *et al.*, 1994; Muller *et al.*, 1993). Most of these techniques, however, did not discriminate between linear and conformational epitopes, or did not associate functional activities with epitopes. Neutralizing linear sites of the MV-H protein have been identified using monoclonal antibodies (MAbs) (Mäkelä *et al.*, 1989*a*; Ziegler *et*

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al., 1996) or anti-peptide antisera (Mäkelä *et al.*, 1989*b*). In the present study, we have developed MAbs which protect against lethal challenge with rodent-adapted measles virus and which recognize a defined linear site. They seem to define a topographically and functionally distinct interface of the H protein.

Methods

■ **Monoclonal antibodies.** MAbs (BH1, BH47, BH59, BH103, BH129; numbers designate sequential clones) were derived from mice immunized with affinity-purified Edmonston B strain MV inactivated with propiolactone, as described (Fournier *et al.*, 1996; Ziegler *et al.*, 1996). Concentrations of the affinity-purified MAbs were determined as absorbance (*A*) at 280 nm. The above MAbs were all of the IgG1 subclass as determined with a commercial kit (Hycult Biotechnology). Western blots were done with concentrated virus, purified by sucrose gradient centrifugation (Fournier *et al.*, 1996).

■ **Peptides.** Synthesis of 121 biotinylated pentadecapeptides overlapping by 10 amino acids and covering the whole sequence of the MV-H protein (Edmonston strain; Alkhatib & Briedis, 1986) and synthesis of free truncation-analogues of peptides H236–250 and H241–255 were performed by simultaneous multiple-peptide synthesis as described (Wiesmüller *et al.*, 1992; Ziegler *et al.*, 1996). Biotinylated peptides were modified C-terminally by two ε-amino caproic acid residues and one lysine residue which separate the peptide from the biotin coupled to the Nε-amino group of lysine amide. ELISAs and competition ELISAs based on the biotinylated peptides were as described (Fournier *et al.*, 1996; Ziegler *et al.*, 1996).

■ **MV-ELISA.** MV-specific antibodies were detected using either an ELISA based on immobilized MV concentrate (Fournier *et al.*, 1996) or a commercial certified ELISA based on permanent monkey kidney cells (Enzygnost; a kind gift of Dr Giesendorf, Behringwerke, Marburg, Germany).

■ **Functional assays.** Neutralization inhibition (NT) was measured in the presence of MAbs using an early passage of MV Edmonston strain (ATCC VR-24) grown on Vero cells as described previously (Norrby & Gollmar, 1972; Muller *et al.*, 1995). Haemagglutination inhibition assays (HI) and haemolysis inhibition (HLI) were measured in a combined assay as described previously (Norrby & Gollmar, 1972, 1975; Orvell, 1976). In brief, 100 µl of MAb was incubated for 1 h at 22 °C with an equal volume of MV in a V-bottom microtitre plate; 50 µl of a 10% suspension of washed African Green monkey (*Cercopithecus aethiops*; RIVM, Bilthoven, The Netherlands) erythrocytes was added and incubated for 3 h at 37 °C. After low speed centrifugation, lysis was assessed in the supernatant as A_{540}. For the rosette forming assay, 10^5 Ltk$^-$ cells transfected with H (Ltk$^-$H; kindly provided by Drs Wild and Beauverger, Lyon, France; Beauverger *et al.*, 1993, 1994) were incubated for 1 h at 4 °C with 200 µl of diluted MAbs. Monkey erythrocytes were then added to a final concentration of 0·1% and incubated for 1 h at 37 °C. Rosette inhibiting titres were defined by the abrupt decrease in number and size of rosettes. Rosettes were defined as cells associated with at least three erythrocytes (Dunster *et al.*, 1994).

■ **Flow cytometry.** A persistently infected human Epstein–Barr virus (EBV)-transformed B cell line (WMPT; gift from Dr Chain, University College Medical School, London, UK) and Ltk$^-$H were used to test the reactivity of the MAbs with MV or MV-H protein by flow cytometry as described (Muller *et al.*, 1995). The FITC-conjugate alone on these cells, as well as MV-free WMPT cells and Ltk$^-$F cells (a gift of Drs Wild and Beauverger; Beauverger *et al.*, 1993, 1994) served as negative controls. In competition experiments between BH47 (IgG1) and BH216 (IgG2b), antibody binding was monitored with a subclass-specific FITC-labelled F(ab′)$_2$ fragment of a goat anti-mouse IgG (Southern Biotechnology Associates).

Results

MAb specificities

After immunization with native MV, H-specific MAbs were obtained which in an ELISA based on biotinylated peptides mapped to amino acids 226–255 (Fig. 1). The MAbs generated a typical band of 80 kDa under denaturing and reducing conditions by Western blot. They reacted both in a certified diagnostic ELISA (Enzygnost), and with immobilized MV purified from culture. In the latter assay, 50%-saturating concentrations (S_{50}%) were: BH1, > 300 ng/ml; BH47, 25 ng/ml (not shown); BH59, 20 ng/ml; BH103, 160 ng/ml; BH129, 30 ng/ml (Fig. 1). The binding capacity of the virus for BH1 appears to be considerably lower than for the other MAbs.

BH1 recognized the pentadecamer H226–240 (S_{50}% > 2000 ng/ml, Fig. 1), but not the free octamer H226–233, in a binding competition ELISA (not shown), indicating that amino acids 233–240 are critical for binding of this MAb. While BH47 (data not shown), BH59 and BH103 recognize peptides H236–250 (S_{50}% 190, 90 and 500 ng/ml respectively) and H241–255 (S_{50}% 180, 90 and 500 ng/ml respectively) equally well, BH129 has a tenfold higher affinity for H241–255 (S_{50}% 35 ng/ml) than for H236–250 (S_{50}% 340 ng/ml). The specificity of the binding has been confirmed by competition with free amide peptides and irrelevant peptides (data not shown).

With the exception of BH1, all MAbs stained MV-infected EBV cells (WMPT-MV) by flow cytometry in comparison with the same cells preincubated with the goat-anti-mouse FITC conjugate alone (Fig. 2 A–D) or MV-free WMPT cells (data not shown). Similar results were obtained with H-transfected Ltk$^-$ cells and Ltk$^-$F cells as negative controls (data not shown).

After intraperitoneal immunization and boosting with peptide H236–250 coupled to KLH (125 µg emulsified in complete/incomplete Freund's adjuvant) sera were obtained which reacted with the native MV as shown by flow cytometry on WMPT-MV cells and appropriate controls (Fig. 2E, F).

These experiments demonstrate that the site associated with H236–250 and H241–255 is expressed on the surface of budding MV and recombinant H protein expressed in eukaryotic cells, while epitope 233–240 recognized by BH1 is perhaps not accessible or the reactivity of this MAb with the native protein is too weak.

Fine mapping

The site shared by peptides H236–250 and H241–255 was further defined using free truncation-analogues to inhibit

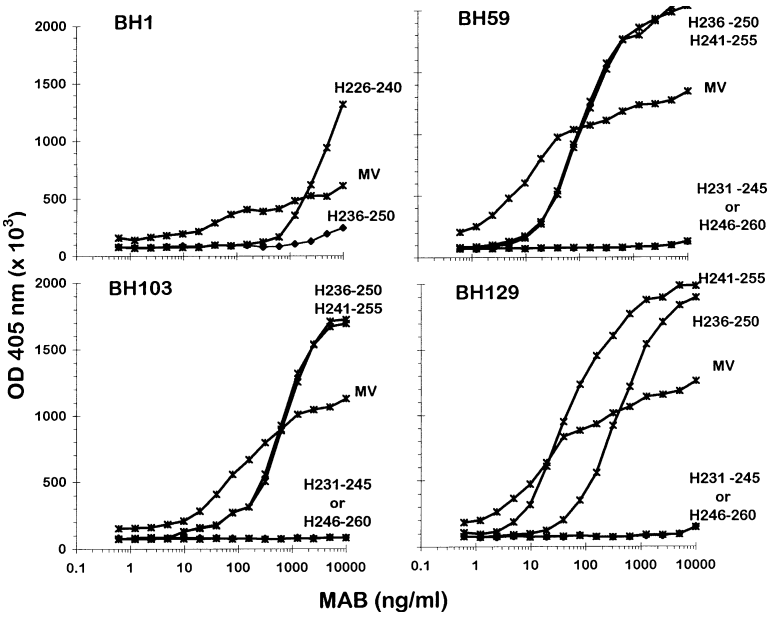

Fig. 1. Titration of affinity-purified MAbs BH1, BH59, BH103 and BH129 against an MV concentrate or the C-terminally biotinylated 15-mer peptides H236–250, H241–255 and H226–240 (only BH1) by ELISA. The titration curves against the peptides H231–245 or H246–260 were invariably negative with BH1 (not shown), BH59, BH103 and BH129 and fully overlapped with each other. The titration curves of BH47 (not shown) are similar to those of BH59. Peptides were selected on the basis of their reactivity in a Pepscan ELISA using biotinylated peptides covering the whole sequence of the MV-H protein (cf. Ziegler *et al*., 1996).

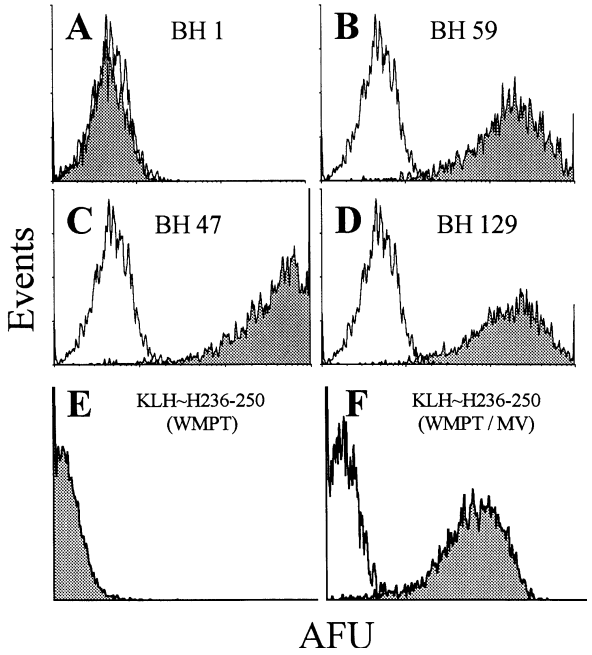

Fig. 2. Binding of MAbs (A–D) and anti-peptide serum (E, F) to MV-infected EBV-transformed human B cells (WMPT/MV; A–D, F) or MV-free WMPT cells (E) measured by flow cytometry. Background binding corresponds to the FITC-conjugated goat anti-mouse IgG alone (A–D) or an irrelevant antibody (E, F). Background binding of MAbs to uninfected EBV cells (WMPT) was similar to the background of the FITC-conjugate alone on MV-expressing cells (data not shown). Panels A–D and E, F represent independent experiments. The data are expressed on a log-scale (3 decades) as arbitrary fluorescence units (AFU).

binding to H241–255 (Fig. 3) and H236–250 (data not shown). The 7-mer H244–250 competed up to five times more efficiently than the free 15-mer, while the 6-mer was a poor inhibitor. Similar results were obtained in competition assays with H236–250.

Functional activities of MAbs

For the following combined HI/HLI assay MAbs were titred against MV by ELISA and concentrations were normalized on the basis of their $S_{50}\%$, corresponding to 1 'IgG unit' (cf. Fig. 1 and Table 1). When HLI and HI were concomitantly monitored in a combined assay, MAbs BH47, BH59 and BH129 inhibited haemolysis with an efficiency similar to haemagglutinin noose epitope (HNE) MAbs (BH6, BH21 and BH216): 13–28 vs 8–24 IgG units (Table 1). Both groups of antibodies, however, markedly differed in their HI capacity: 500– > 5800 IgG subunits in comparison to 20–45 IgG subunits for the HNE MAbs (Table 1). In the case of the anti-HNE MAbs (BH6, BH21 and BH216), the $IC_{50}\%$ for lysis corresponds to the concentration for HI, while BH47, BH59 and BH129 show only residual HI activity, if any, at the concentration at which fusion inhibition is complete (Table 1). Thus, in contrast to other H-specific MAbs, which inhibit HLI by blocking virus binding (HI^+), antibodies to site 241–250 inhibit HLI in a more direct way (HI^-). Also, 10 to > 200 times higher concentrations of mAbs BH47 (4·2 μg/ml), BH59 (55 μg/ml), BH129 (> 23 μg/ml) are required, in comparison with HNE MAbs (0·25–0·43 μg/ml), in order to inhibit rosette formation with monkey erythrocytes and Ltk^-H cells (data not shown).

Challenge/protection experiments

Single doses of purified BH47 (30–315 μg), BH59 (66–315 μg) and BH129 (66–315 μg) were injected intraperitoneally into 3-week-old CBA mice, which were challenged 2 days later by intracerebral injection of a lethal dose (12500 $TCID_{50}$) of CBA-adapted MV (derived from the CAM/RB strain; a gift from Dr Liebert, Leipzig, Germany; Liebert & ter Meulen, 1987). These MAbs gave full protection

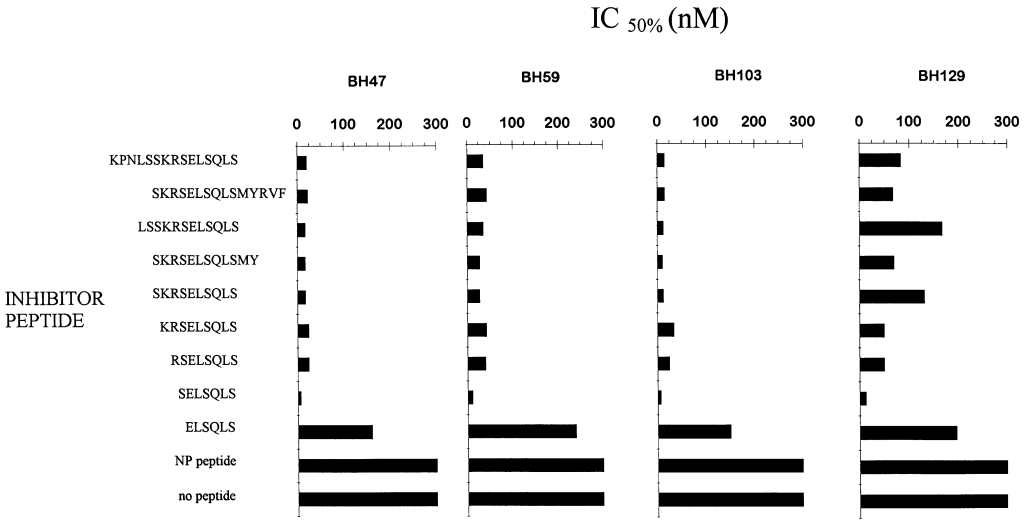

Fig. 3. Inhibition of binding of saturating concentrations of MAb to peptide H241–255 (cf. Fig. 1) by truncation-analogues. Data are expressed as the concentration of soluble free inhibitor peptide required for 50% inhibition ($IC_{50\%}$) of binding of MAbs to immobilized biotinylated peptide H241–255. $IC_{50\%}$ was truncated at 300 nM. At this highest concentration tested, competition with irrelevant NP peptide or no peptide showed less than 5% inhibition.

Table 1. Functional characteristics of MAbs of the HNE domain (381–400; Ziegler *et al.*, 1996) and the H244–250 site

Epitope	MAb	MV-ELISA ($S_{50\%}$)*	NT (IgG units)†	HI‡ (IgG units)	HLI‡§ (IgG units)
H381–400	BH6	13	430	32	20
H381–400	BH21	8	500	20	8
H381–400	BH216	12	650	45	24
H244–250	BH47	25	240	4100	28
H244–250	BH59	20	440	> 5800	14
H244–250	BH129	30	630	500	13

* $S_{50\%}$, 50% saturating concentration in MV-ELISA in ng/ml.
† 1 IgG unit corresponds to the $S_{50\%}$ (ng/ml) (cf. column 3).
‡ Combined HLI/HI assay.
§ HLI titre corresponds to 50% haemolysis.

even at the lowest concentrations tested, whereas MAb BH1 did not at 20 times this concentration (Fig. 4).

Binding competition experiments between MAbs

To examine the topographical relationship between the linear site defined here and the HNE region (Ziegler *et al.*, 1996) binding competition experiments were done on MV-infected cells (WMPT) and on Ltk$^-$H cells using MAbs BH47 (IgG1) and BH216 (IgG2b) and IgG-subclass-specific FITC conjugates. Fig. 5 shows that binding of BH47 cannot be blocked by increasing concentrations of BH216 and BH216 cannot be blocked by BH47. No differences were found between WMPT and Ltk$^-$H cells. The HI activity of BH216 is not modified by increasing concentrations of BH47, while the HLI activity of these two MAbs is additive (data not shown). Thus these two binding sites represent non-overlapping, independent domains, corresponding to different functional interfaces of the H protein.

Discussion

We developed a series of MV-specific MAbs which reacted with MV by Western blot under reducing conditions, neutralized the virus *in vitro* and protected mice against lethal challenge with a rodent-adapted MV strain. The antibodies

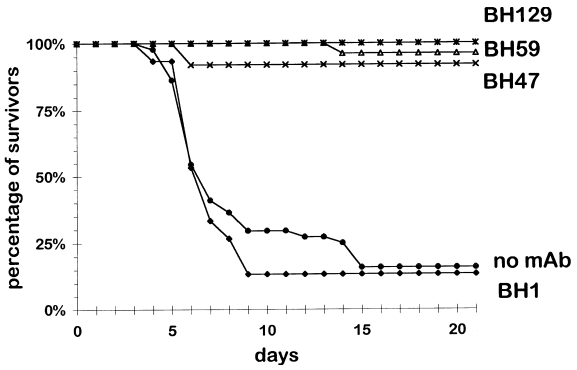

Fig. 4. Challenge protection experiment with mice passively immunized on day −1 with BH47 [315 μg (11 mice); 100 μg (10 mice); 30 μg (4 mice)], BH59 [315 μg (9), 200 μg (7), 100 μg (5), 66 μg (5)] and BH129 [316 μg (4), 100 μg (5), 66 μg (5)] or 630 μg BH1. On day 0 mice were challenged with an intracranial injection of a mouse-adapted MV. Naive mice were challenged without prior antibody transfer. Each survival curve sums the data from three or four independent experiments. 315 μg of BH103 protected 4/4 mice (data not shown). None of the mice treated with the lowest concentrations died.

were mapped to a linear site corresponding to two 15-mer peptides (H236–250 and H241–255) sharing the amino acids S^{241}KRSELSQLS^{250} of the MV-H protein. With truncation-analogues the site was further reduced to a 7-mer, S^{244}ELSQLS^{250}. Neutralizing epitopes have previously been located between amino acids 200 and 370 by studying the 'footprints' of MAbs (I-41, 7-AG11, I-29 and I-44; Sheshberadaran & Payne, 1988) and the sequences of escape mutants (I-29, amino acids 313/314; 16DE6, three amino acid changes, one of which is amino acid 211; Sheshberadaran & Norrby, 1986; Hu *et al.*, 1993; Liebert *et al.*, 1994). No such mutation has previously been found in the site described here. In a wild-type isolate (JM-1977) with mutations in position 211 and 243, reactivity of MAb 79-XV-V17 was lost (Tamin *et al.*, 1994; Rota *et al.*, 1992). Studies with chimeric proteins have located the binding site of MAb 16-CD-11 to amino acids 211–291 (Hummel & Bellini, 1995). Human late convalescent sera did not react with peptides representing this region and reactivity of mouse and rabbit hyperimmune sera was variable (Muller *et al.*, 1993; Obeid *et al.*, 1994).

Both H and F protein are required for fusion (Wild *et al.*, 1991). MV-H is responsible for cell tropism while MV-F is interchangeable with F protein of other morbilliviruses (Stern *et al.*, 1995). The primary function of the MV-H protein is to mediate virus attachment to CD46 of the target cell (Naniche *et al.*, 1993), which is followed by fusion of the viral envelope with the cell membrane (Gerlier *et al.*, 1995). This sequence of events may explain why H-specific antibodies, which inhibit haemagglutination (i.e. virus binding), also prevent haemolysis (i.e. fusion) indirectly. When HI MAbs were removed from the serum by adsorption with Tween–ether-treated MV (Norrby & Gollmar, 1975), the remaining HLI activity was mostly MV-F specific. Also, MV-F-specific MAbs are typically HLI^+ and HI^-. The MAbs corresponding to the site S^{244}ELSQLS^{250} are remarkable in that they are also HI^- and HLI^+, but they are MV-H specific. Another antibody (I-29) with these properties has been mapped to the epitope 309–318 (Mäkelä *et al.*, 1989*a*) with critical amino acids in position 313/314 (Hu *et al.*, 1993). Such MAbs inhibit the functional cross-talk between the H and F proteins in a more direct way than HI^+ MAbs. Mechanisms of action may include the prevention of conformational or topographical rearrangements of the H protein or simply steric effects.

Data from escape mutants (Sheshberadaran & Norrby, 1986; Hu *et al.*, 1993; Liebert *et al.*, 1994), even when complemented with MAb competition experiments (Carter *et al.*, 1982; Mäkelä *et al.*, 1987), cannot unequivocally define antibody-binding domains (Diamond *et al.*, 1985; Parry *et al.*, 1990). Binding assays with chimeric and deletion homologues of the H protein do not normally have the sensitivity of those with short peptides (Hummel & Bellini, 1995). Peptide mapping of epitopes may offer some advantages over other techniques, although it is normally restricted to a few sequential epitopes. Here, we show that MAbs binding to (HI^-/HLI^+) site 244–250 are functionally different from those binding to (HI^+/HLI^+) domain 381–400, recently described by us (Ziegler *et al.*, 1996). Moreover, the functionally distinct MAbs specific for the two

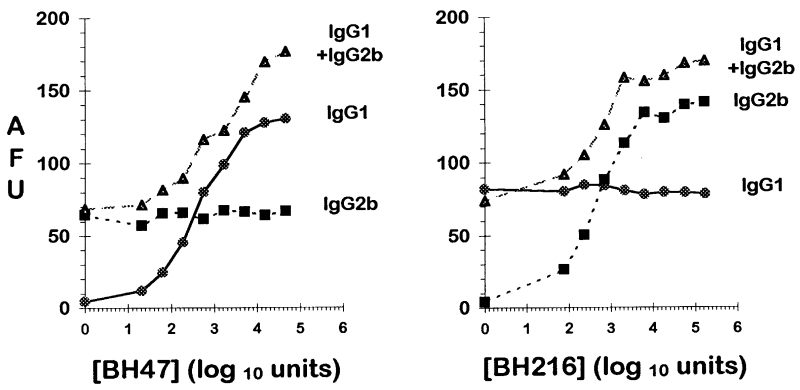

Fig. 5. Binding competition assay of BH47 (IgG1) and BH216 (IgG2b) on MV-superinfected EBV-transformed human B cells measured by flow cytometry. Data are expressed in mean arbitrary fluorescence units (AFU) of 5000 cells. Cells were incubated with a constant saturating concentration of BH216 (left panel) or BH47 (right panel) in the presence of increasing concentrations of BH47 (left panel) or BH216 (right panel). Subclass-specific FITC-conjugates were used as indicated to detect MAb binding. MAb concentrations are expressed in units of specific IgG. One IgG unit corresponds to the $S_{50\%}$ concentration.

sites bind to topographically non-overlapping domains. They seem to define two functionally and/or topographically distinct interfaces of the H protein, one of which is clearly not involved in the H/receptor interaction. An attractive interpretation of these observations would be that this interface is biased towards a functional and/or topographical interaction with the fusion protein. Fusion is dependent on the formation of the H/F/CD46 complex (Wild *et al.*, 1991), suggesting that fusion may be triggered by H/CD46 interaction (Lecouturier *et al.*, 1996). In this context it is interesting that Arg-243, which is directly adjacent to the epitope, is involved in down-regulation of CD46 binding (Bartz *et al.*, 1996; Lecouturier *et al.*, 1996). This could be indicative of a functional link between an H/receptor and an F/H interface.

N-Glycosylation has also been implicated in the cell fusion process (Malvoisin & Wild, 1994). The $S^{244}ELSQLS^{250}$ epitope is downstream of the cluster of glycosylation sites (amino acids 168, 187, 200, 215 and 238; Hu *et al.*, 1994). MAb BH1 reacts with peptides containing the sequence 233–240 but not with the MV-infected EBV-transformed cells or with a eukaryotically expressed recombinant H protein. Glycosylation in position 238 could be one explanation for this finding, although this site was thought not to be utilized, at least in some strains (Hu *et al.*, 1994).

Although the site of virus interactions with the simian or mouse brain receptor may differ from the site interacting with human CD46 (Naniche *et al.*, 1993; Shibahara *et al.*, 1994), the mechanism of haemolysis of monkey erythrocytes and MV infection of human cells may call on similar interactions between the F and H protein. If this is the case, our findings suggest that prevention of H from functionally interacting with F inhibits virus infection both *in vitro* (by neutralization) and *in vivo* (by challenge/protection). Although expected, to the best of our knowledge, this has not been shown *in vivo* with any other HI^-/HLI^+ H-specific MAb. It means that HI activity and protection are not necessarily linked as has been suggested (Giraudon & Wild, 1985). Segregation of protection and HI activity of MAbs has been observed for other paramyxoviruses (Tsurudome *et al.*, 1986; Piga *et al.*, 1990). Since the MAbs are of the IgG1 isotype, protection seems to be independent of complement fixation and FcγRI binding (Neuberger & Rajewsky, 1981; Ravetch & Kinet, 1991), the most important effector mechanism in virus clearance (Ishizaka *et al.*, 1995).

Site 381–400 was referred to as HNE381–400 (Haemagglutinin Noose Epitope or Haemagglutinating and Neutralizing Epitope; Ziegler *et al.*, 1996). To discriminate between epitopes involved in HI or not, epitope 244–250 could be referred to as NE244–250 (Neutralizing Epitope only).

The site described here is an attractive candidate to incorporate into a subunit vaccine. It is linear and its antigenicity and immunogenicity can be mimicked by short peptides. The experience with the killed MV vaccine facilitating atypical measles suggested that antibodies against the fusion protein are required for full protection (Norrby *et al.*, 1975; Fulginiti *et al.*, 1967). Although antibodies against the site described here are H-specific, they exhibit the main properties (HI^-, HLI^+) of F-specific antibodies. Two protective sequences of the F protein have been described (Steward *et al.*, 1995; Obeid *et al.*, 1995), but whether in the absence of epitopes of the F protein, abundant antibodies against the NE epitope could potentially be sufficient to prevent atypical measles is a challenging question.

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