Probing neutralizing-antibody responses against emerging measles viruses (MVs): immune selection of MV by H protein-specific antibodies?

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Measles virus (MV) infection and vaccination induce long-lasting immunity and neutralizing-antibody responses that are directed against the MV haemagglutinin (H) and the fusion (F) protein. A new MV genotype, D7, emerged recently in western Germany and rapidly replaced the long-term endemically circulating genotypes C2 and D6. Analysis of the H gene of C2, D6, D7 and vaccine viruses revealed uniform sequences for each genotype. Interestingly, a consistent exchange of seven distinct amino acids in the D7 H was observed when compared with residues shared between C2, D6 and vaccine viruses, and one exchange (D416R) in the D7 H was associated with an additional N-linked glycosylation. In contrast, the F gene is highly conserved between MVs of these genotypes. To test whether the D7 H protein escapes from antibody responses that were raised against earlier circulating or vaccine viruses, the neutralizing capacity of mAbs recognizing seven distinct domains on the H of an Edmonston-related MV was compared. The mAbs revealed a selective and complete loss of two neutralizing epitopes on the D7 H when compared with C2, D6 and vaccine viruses. To assess whether these alterations of the D7 H affect the neutralizing capacity of polyclonal B-cell responses, genotype-specific antisera were produced in cotton rats. However, no significant genotype-dependent difference was found. Likewise, human sera obtained from vaccinees (n = 7) and convalescents (n = 6) did not distinguish between the MV genotypes. Although the hypothesis of selection of D7 viruses by pre-existing neutralizing antibodies is compatible with the differing pattern of neutralizing epitopes on the H protein, it was not confirmed by the results of MV neutralization with polyclonal sera.

INTRODUCTION

Measles virus (MV) infection and vaccination with a live-attenuated vaccine induce long-lasting immunity. Protection against measles is mediated both by antibodies and by T-cell immunity. Neutralizing-antibody responses are directed solely against the viral surface glycoproteins, the haemagglutinin (H) and the fusion (F) protein. The H protein is responsible for attachment of the virion to host-cell receptors (Döring et al., 1993; Naniche et al., 1993; Wild & Buckland, 1995; Tatsuo et al., 2000; Erlenhöfer et al., 2001; Schneider-Schaulies et al., 2001) and acts in concert with the F protein during fusion and virus entry (Wild et al., 1991). Neutralizing antibodies inhibit virus infection by preventing the interaction of the H protein with its cell receptors and by blocking fusion activity (Bouche et al., 2002).

Wild-type MVs are currently divided into 22 genotypes, based on sequences derived from the N and H genes (WHO, 2003). MV is considered to be antigenically stable and serologically monotypic. Epidemiological observations have provided evidence that measles immune responses protect against (re)infection, irrespective of the genotype of the
circularizing virus. Vaccine virus strains originating from the same progenitor of genotype A have been used successfully throughout the world over a 30 year period.

In Germany, MV circulation has been monitored in an area-wide and continuous manner since 1999. Endemic transmission was associated with genotypes C2, D6 and D7. In western Germany, the incidence of measles indicated high endemic transmission (9–30 cases per 100 000 population during the years 2000 and 2001), whilst the simultaneous disappearance of genotypes C2 and D6 and the concomitant increase in the number of D7 cases detected in the years 2000, 2001 and 2002, respectively, revealed an increasing proportion of genotype D7, from 45% in 2000 to 91% in 2001 and 98% in 2002. Before D7 viruses emerged, C2 and D6 viruses co-circulated for a period of at least 8 years in Germany and were distributed widely over Europe. Comparative analysis of the H gene revealed an exchange of seven amino acid residues in the D7 H protein (Santibanez et al., 2002). They are located at positions 174 (T→A), 176 (T→A), 195 (R→K), 296 (L→F), 302 (G→R), 308 (I→V) and 416 (D→N) in β-sheets 6, 2 and 3, according to the measles H structure model (Langedijk et al., 1997; Vongpunsawad et al., 2004). The exchanges at positions 195, 296, 302 and 308 are in regions that are proposed to be epitopes recognized by H-specific mAbs (Bouche et al., 2002). At asparagine residue 416, the D7 H has an additional potential site for N-linked glycosylation that seems to be restricted to viruses of more contemporary circulating genotypes (Rota et al., 1992; Saito et al., 1995; Kubo et al., 2003). As the H protein is the prime target for neutralizing and protective antibodies (Giraudon & Wild, 1985; Varsanyi et al., 1987), the question arises whether the amino acid exchanges are facilitating an escape of D7 viruses from antibody responses. The assumption that currently circulating MVs are exposed to immune selection by pre-existing neutralizing antibodies is supported by recent data indicating that different wild-type viruses are discriminated by human antibody responses (Klingele et al., 2000).

The observation that, in isolated populations, measles vaccinees exhibited secondary immune responses (SIR) in the absence of measles (Pederson et al., 1989; Edmonson et al., 1990) indicated that MV could survive and circulate in fully protected vaccinated individuals undergoing asymptomatic recurring infection (Muller et al., 1996; Damien et al., 1998). Among individuals protected fully against disease, those prone to asymptomatic SIR are the most likely to support subclinical MV transmission (Damien et al., 1998). Susceptibility to subclinical reinfection is essentially higher in vaccinees than in late convalescents (Damien et al., 1998), suggesting that susceptibility to SIR is increasing in highly vaccinated populations. Isolation of MV from asymptptomatically reinfecced individuals has been reported (Vardas & Kreis, 1999; van Binnendijk et al., 2003). During reinfection, MV with lower susceptibility to pre-existing neutralizing antibodies might have an advantage in terms of replication efficiency, resulting in competitive exclusion of C2 and D6 viruses by D7 viruses.

The present study addressed the question of whether the newly emerged D7 viruses are less susceptible to the neutralizing capacity of polyclonal B-cell responses than the previously circulating C2 and D6 viruses. We demonstrate that the pattern of glycosylation and neutralizing epitopes in the H protein clearly differs between D7 viruses and C2, D6 and vaccine viruses. In contrast, the neutralizing capacity of pre-existing antibodies in sera from MV-infected cotton rats and vaccinated and convalescent individuals is independent of the MV genotype.

**METHODS**

MV strains and isolates used in this study are described in Table 1.

**Nucleotide sequence determination of the MV F protein-coding region.** Viral RNA of MV isolates C2-a, C2-b, D6-a, D7-d and D7-f was reverse-transcribed using primer MeF1 (nt 5371–5391, 5′-CAATCCACCAACAYCCGACAGG (Y=C/T)) (nucleotide positions are according to Parks et al., 2001) and reverse transcriptase Superscript II (Invitrogen). A fragment containing the F protein-coding region was amplified from cDNA using primers MeF1 and MeF2 (nt 7178–7159, 5′-GGTGCGGTGGTTGCTTGATG) and TaKaRa La Taq polymerase. Amplified DNA was sequenced with an ABI Prism BigDye Terminator cycle sequencing kit (Applied Biosystems, Perkin Elmer) using forward primers MeF3 (5′-CTAAAGGAGACACCCCGGAATC) and MeF8 (5′-GATGCACATATGGCAGTAC), MeF11 (5′-GGCGGAGATCTCATCCGAG), MeF15 (5′-CCAGGGTACACCTTATC), MeF16 (5′-GCAAATGTTAACACAG- AGGAACG) and reverse primers MeF4 (5′-CGAAGGAGAGCTTGGGAGCC), MeF10 (5′-ACTCCCAAGACACACTCG), MeF17 (5′-GTATGTAGGATCTT- GTGCAGG), MeF18 (5′-GTATTTGTCAGGAGAGCC), MeF19 (5′-CTCTCTTGTGGTCTCC) and MeF6 (5′-CTGCGCAATATGTGGCAG).

**Cloning the coding region of the MV H gene.** Viral RNA of MV isolates D6-a and D7-d was reverse-transcribed using the primer MeH1 (nt 7214–7233, 5′-CTCCTGCGCGACAATATCG) (nucleotide positions are according to Parks et al., 2001) and reverse transcriptase Superscript II (Invitrogen). A fragment containing the sequence encoding the MV H protein (H-cDNA) was amplified from cDNA by PCR using the forward primer MeH1 (nt 7271–7291, 5′-ATGTCACCAACAAAGCAG) and the MV genotype-specific reverse primer MeH2-D6 (nt 9124–9104, 5′-CTATCCACCACTGAGGTTTTCATC) or MeH2-D7 (nt 9124–9104, 5′-CTATCCACCACTGAGGTTTTCATC) and TaKaRa LA Taq polymerase. Amplified H-cDNA was cloned by using a TOPO TA cloning kit dual promoter with the vector pCR II TOPO (Invitrogen) and One Shot TOP10 chemically competent Escherichia coli (Invitrogen). Plasmids containing full-length insertions of H-cDNA were sequenced and one of six clones derived from the D6 virus isolate and one of seven clones derived from the D7 virus isolate were selected for generation of MV expressing recombinant vaccinia viruses (rVs). Selected clones encode a primary structure identical to that predicted from the H sequence that was determined previously for the corresponding virus isolates (Santibanez et al., 2002). H-cDNA-containing fragments were subcloned into a SalI- and HindIII-cleaved p7:Sk131A vector.
Generation of recombinant rVVs. rVVs were constructed as described previously (Hengel et al., 1997). Briefly, p7.5K131A constructs containing H-cDNA were used to generate rVVs after homologous recombination with the VV strain Copenhagen. rVVs expressing the H gene of MVs D6-a (rVV-H/D6) and D7-d (rVV-H/D7) were isolated by selection with 5-bromo-2′-deoxyuridine using tk-143 cells.

Metabolic labelling, immunoprecipitation and endoglycosidase H (Endo H) treatment of MV H protein. Immunoprecipitation of proteins was performed as described previously (Hengel et al., 1995). In brief, CV1 (green monkey kidney) cells were infected with rVV-H/D6 or rVV-H/D7 at an m.o.i. of 3. At 16 h post-infection, cells were washed and exposed to methionine- and cysteine-free medium for 1 h before [35S]methionine and -cysteine (Amersham Biosciences) were added at a concentration of 500 μCi (18.5 MBq) ml⁻¹ for 1 h. After washing with PBS, cells were lysed in 1 ml 1 % NP-40-containing lysis buffer as described previously (Hengel et al., 1995). Cellular lysates were centrifuged at 13 000 g for 30 min to remove nuclei and debris. Hybridoma supernatant containing mAb HA 55 (Giraudon & Wild, 1981) (200 μl) was added to the lysates for 1 h at 4 °C. Immune complexes were adsorbed onto protein G-Sepharose (Amersham Biosciences) during incubation for 1 h at 4 °C, washed and eluted by boiling for 5 min in sample buffer as described previously (Hengel et al., 1995). Aliquots of precipitated H proteins were digested with 0.5 mU Endo H (Roche) at 37 °C for 3 or 30 min or for 1, 3 or 6 h or with 20 mU Endo H for 24 h. After boiling for 5 min with an SDS- and 2-mercaptoethanol-containing sample buffer, proteins were analysed by 8–10 % gradient SDS-PAGE.

Generation of mAbs. MV H-specific mAbs (BH6, BH15, BH17, BH47, BH67, BH81, BH97, BH125 and BH141; numbers designate sequential clones) were derived from mice immunized with MV Edmonston B, as described previously (Fournier et al., 1996; Ziegler et al., 1996). The mAbs were characterized by an ELISA based on biotinylated overlapping peptides (mAbs BH6 and BH47; Fournier et al., 1997) and by investigation of the competition pattern (mAbs BH15, BH97, BH125, BH17, BH1141, BH67 and BH81; Bouche et al., 2002). Purification of mAbs was performed by protein G affinity chromatography.

Plaque-reduction neutralization test (PRNT) using mAbs. Solutions of MV H-specific mAbs were diluted by f4 × 10² (f4, mAb-specific dilution factor; n = 1, 2, 3, 4, 5 and 6) in minimal essential medium (MEM) alpha medium (Invitrogen) supplemented with 5 % fetal calf serum (FCS). Mixtures of MV and mAb were prepared by adding a mAb dilution to an equal volume of an MV suspension containing 40–60 p.f.u. in 100 μl and incubated for 60 min at 37 °C. Aliquots (100 μl) of these mixtures were transferred into cell-culture wells with a confluent monolayer of signalling lymphocytic activation molecule (SLAM)-transduced CHO cells and incubated at 37 °C for 60 min. The inoculum was removed and the monolayers were covered with an overlay containing 0.5 % carboxymethylcellulose and 3 % FCS and incubated for 3 days. The monolayers were stained with crystal violet and fixed with 3 % formalin. Plaques were counted visually. For a given MV/mAb pair, each test was performed in three replicate cultures and repeated at least twice. The plaque number of a given virus in the absence of antibody was determined in each experiment. The amount of IgG per well that resulted in 50 % reduction of the plaque number was calculated.

Polyclonal sera from MV-infected cotton rats. Cotton rats (inbred strain COTTON/Nico) were obtained from Iffa Credo, France. Animals were kept under controlled conditions and used at the age of 6–8 weeks (60–70 g). Intraperitoneal infection was carried out under ether narcosis by injecting 1–5 × 10⁴ TCID₅₀ of the respective MV (vaccine strains Edmonston Zagreb and Leningrad 16;
isolates C2-a, C2-b, C2-c, D6-a, D7-a, D7-c and D7-d) at 2-weekly intervals twice into cotton rats. Four weeks after the first immunization, cotton rats were sacrificed by CO₂ inhalation and exsanguinated by heart puncture.

Human sera. Sera from vaccinated, late-convalescent and early-convalescent individuals were used in PRNT. Data describing these sera are provided in Table 2.

PRNT using polyclonal sera. Cotton-rat and human sera were diluted by 50⁶²ⁿ (n = 1, 2, 3, 4, 5, 6 and 7) in MEM alpha medium (Invitrogen) supplemented with 5 % FCS. For pairs of MV and serum, PRNT was performed as described above for MV/mAb pairs. Generally, an MV/serum pair was retested three times and PRNT titres (serum titre that resulted in 50 % reduction of plaque number) were calculated. Based on PRNT titre data, geometric mean serum titres (GMTs) that summarized the data obtained for the individual virus isolates on the genotype level were calculated. GMTs obtained for a given serum from two different genotypes were compared by Student's test using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA). Statistical significance of differences was defined as P < 0.05.

RESULTS

The F protein is highly conserved between MVs of genotypes C2, D6 and D7

The action of neutralizing antibodies is directed against both viral surface glycoproteins H and F, which collaborate during fusion and virus entry (Wild & Buckland, 1995). Therefore, the primary structure of the H and F proteins was compared between the newly emerged D7 viruses and the previously circulating C2 and D6 viruses. Previous analysis of H showed an exchange of seven amino acid residues in the D7 H compared with C2, D6 and vaccine viruses. They are located at positions 174 (T → R), 176 (T → A), 195 (R → K), 296 (L → F), 302 (G → R), 308 (I → V) and 416 (D → N) (Santibanez et al., 2002).

In this study, the sequence of F was determined for MVs of genotypes C2, D6 and D7 (Table 1). Three potential AUG start codons are present in the F mRNA of C2 and D6 viruses. In the F mRNA of D7 viruses, the first of these codons is exchanged for the codon GUG. Comparison of the resulting maximum ORF shared between C2, D6 and D7, which would encode 550 aa, revealed a very high degree of conservation. The D7 F showed a single exchange (G → D) at the second position only, whereas the D6 F differs at positions 5, 9 and 14 from C2 and D7. With exception of these exchanges, sequence identity was found in the F protein between MVs of genotypes C2, D6 and D7. Based on the primary structure data predicted for H and F, we conclude that alterations of the antigenic structure might have occurred in the H rather than in the F protein and, therefore, further investigation is focused on the H protein.

An additional N-linked glycan is attached to the D7 H protein

Comparison of the predicted amino acid sequences of the H protein indicated that MVs belonging to genotypes C2, D6 and D7 share five potential N-linked glycosylation sites, located at residues 168, 187, 200, 215 and 238. These glycosylation sites were also found in the Edmonston
strain, with the first four of them being used (Cattaneo & Rose, 1993). The H sequence of the newly emerged D7 viruses predicts an additional potential site of N-linked glycosylation at position N416 compared with the previously circulating C2 and D6 viruses and the Edmonston virus (Santibanez et al., 2002). To confirm the presence of the supposed additional carbohydrate chain, the H protein of MV isolates D6-a and D7-d was expressed by rVV (rVV-H/D6, rVV-H/D7) in CV1 cells, immunoprecipitated from metabolically labelled cell lysates, treated with Endo H and analysed by SDS-PAGE. Partial Endo H digestion patterns of the D7 H protein (on the left) and the D6 H protein (on the right) are shown. The position of the 69 kDa molecular mass marker is indicated on the right. The duration (min) of Endo H treatment for each sample is shown above each lane. In addition to the completely deglycosylated protein band, indicated as 0, four intermediates for the D7 H and three intermediates for the D6 H can be detected, besides fully glycosylated H proteins.

**Fig. 1.** An additional N-linked glycan is attached to the D7 H protein. Lysates were prepared from [35S]methionine-labelled CV1 cells that were infected with the recombinant vaccinia viruses rVV-H/D7 and rVV-H/D6. H proteins were immunoprecipitated with mAb HA 55 and the immune complexes were digested with Endo H and analysed by SDS-PAGE. Partial Endo H digestion patterns of the D7 H protein (on the left) and the D6 H protein (on the right) are shown. The position of the 69 kDa molecular mass marker is indicated on the right. The duration (min/h) of Endo H treatment for each sample is shown above each lane. In addition to the completely deglycosylated protein band, indicated as 0, four intermediates for the D7 H and three intermediates for the D6 H can be detected, besides fully glycosylated H proteins.

mAbs reveal a selective loss of epitopes on the D7 H protein

To study whether the D7 H protein has an altered pattern of neutralizing epitopes, the neutralizing capacity of nine anti-H mAbs binding to seven distinct sites of the H protein (Ziegler et al., 1996; Fournier et al., 1997; Bouche et al., 2002) was estimated by PRNT. PRNT was performed on SLAM-transduced CHO cells (Erlenho¨fer et al., 2001, 2002), which can be infected by wild-type and vaccine MVs. Two MV isolates of each of the genotypes C2, D6 and D7 and the vaccine virus Edmonston Zagreb were characterized by the mAbs (Table 3). Five of the mAbs (BH6, BH17, BH67, BH81 and BH141) neutralized all MVs investigated. mAb BH97 neutralized C2 and D6 viruses and Edmonston Zagreb, demonstrating the absence of a neutralizing epitope on the D6 H. This mAb was not able

Table 3. Neutralizing capacity of anti-H mAbs

The neutralizing capacity of the mAbs was determined by PRNT on SLAM-expressing CHO cells. Values indicate concentration of IgG (ng in 100 μl) required for 50% reduction of plaque number.

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to precipitate the recombinant D6 H protein, indicating that it does not recognize the D6 H (data not shown). mAbs BH15 and BH125 did not neutralize the D7 viruses, but neutralized C2 and D6 viruses and Edmonston Zagreb. Neither mAb was able to precipitate the recombinant D7 H protein, indicating that they do not recognize the D7 H (data not shown). These observations demonstrate a loss of two neutralizing B-cell epitopes on the H protein of D7 viruses, compared with C2 and D6 viruses and the vaccine virus.

Polyclonal sera from MV-infected cotton rats do not discriminate between D7 and replaced genotypes

We assessed whether the altered antigenic properties of the D7 H protein compared to C2, D6 and the vaccine virus would influence the neutralizing capacity of polyclonal B-cell responses upon MV infection. Cotton rats (Sigmodon hispidus), shown to be useful for experimental MV infection (Niewiesk et al., 1997, 2000; Niewiesk, 1999; Pfeuffer et al., 2003), were infected with MVs of genotypes C2, D6 and D7 (Table 1) and the vaccine viruses Edmonston Zagreb and Leningrad 16. The capacity of the sera to neutralize MVs of different genotypes was determined. The MVs tested in PRNT were the same as those used initially for infecting the animals. Each serum neutralized all MVs tested. For a given serum, the data obtained from the individual MV isolates were grouped and summarized according to genotype (Fig. 2). Individual sera had differing abilities to discriminate between MV genotypes. For example, the serum of the C2-a-infected animal neutralized MVs of all genotypes tested with very similar efficiencies, whereas the anti-C2-c serum partially discriminated between these genotypes. Interestingly, none of the animal sera neutralized D7 viruses less efficiently ($P \geq 0.063$) than C2 and D6 viruses. The anti-Leningrad 16 and anti-D7-d sera had a significantly higher capacity ($P \leq 0.041$) to neutralize D7 viruses compared with C2 and D6 viruses. The data indicate that the antigenic diversity of the H protein, found by the use of mAbs, between genotype D7 and genotypes C2 and D6 does not significantly affect the neutralizing capacity of polyclonal B-cell responses in cotton rats.

Unaltered capacity of human sera from vaccinees and convalescents to neutralize D7 viruses

It has been shown that human sera from vaccinated and convalescent individuals neutralize different wild-type MVs with differing efficiency (Klingele et al., 2000). To elucidate whether human sera can distinguish between the newly emerged D7 viruses, the previously circulating C2 and D6 viruses and the earlier circulating viruses of genotype A, the capacity of human sera to neutralize MVs of these genotypes was estimated by PRNT. Sera were collected from seven vaccinees, three late convalescents and three early convalescents (Table 2). The vaccine viruses Edmonston Zagreb and Leningrad 16, the genotype A viruses Loss and Havlova and viruses of genotypes C2, D6 and D7 (Table 1) were tested against these sera.

Each human serum neutralized all MVs tested. As expected, the neutralizing capacity of sera from vaccinees was lower, on average, than that of sera from convalescent individuals. However, a given serum neutralized individual MV isolates of the same genotype to differing extents. For a given serum,
individual virus isolates were tested and the data obtained were summarized according to genotype (Fig. 3). The sera from vaccinees V1, V2, V3, V5 and V7 neutralized the viruses of the recent genotypes, C2, D6 and D7, with similar efficiency ($P \leq 0.125$). The sera from vaccinees V1, V3, V5, V6 and V7 neutralized the genotype A wild viruses more efficiently ($P \leq 0.003$). The sera from convalescents neutralized the wild viruses of genotypes A, C2, D6 and D7 with similar efficiency ($P \geq 0.062$) (in the case of the early convalescents, genotyping of MVs that were detected during acute infections revealed the presence of D7 viruses). None of the human sera neutralized D7 viruses less efficiently ($P \geq 0.115$) than C2 and D6 viruses. These data suggest that the neutralizing capacity of polyclonal B-cell responses in humans is independent of the MV genotype when comparing D7 with C2 and D6.

**DISCUSSION**

This study investigates whether the currently observed rapid replacement of the previously long-term circulating MV genotypes C2 and D6 by the newly emerged genotype D7 is due to immune selection by neutralizing antibodies. The D7 viruses differ from the replaced C2 and D6 viruses and the vaccine viruses in the surface protein H by seven amino acid residues, including an additional site for N-linked glycosylation. In contrast, the surface protein F, which also exhibits binding sites for neutralizing antibodies (Fayolle et al., 1999), was shown to be highly conserved among MVs of these genotypes. Anti-H mAbs revealed a loss of two neutralizing epitopes on the D7 H protein when compared with C2, D6 and the vaccine virus. However, the altered antigenic properties of the H protein did not affect the neutralizing capacity of polyclonal B-cell responses in MV-infected cotton rats. Likewise, human sera obtained from vaccinated and convalescent individuals did not distinguish between D7 and the previously circulating C2 and D6 viruses.

The fact that vaccine strains that were originally derived half a century ago still provide protection against acute MV infection supports the notion of limited antigenic diversity of MV. However, the introduction and continuously increasing worldwide application of vaccination for over 30 years have rapidly changed the circulation conditions for wild-type MVs. There is evidence that MV may survive and circulate in subclinically infected seropositive individuals showing SIR (Muller, 2001). As the MVs used as vaccines worldwide are genetically closely related, circulating wild-type MVs are increasingly exposed to a directed selective pressure in a population with an increasing proportion of immunized individuals. This might result in changed antigenic properties of wild-type viruses when compared with vaccine viruses.

In Germany, a rapid replacement of long-term circulating MV genotypes by a newly emerged genotype was observed in a region where the vaccination rate was not high enough to prevent endemic virus circulation. Interestingly, the seven amino acid residues in the H protein of the replaced C2 and D6 viruses that are exchanged in the H of the newly emerged D7 viruses are also shared by the vaccine viruses. Remarkably, four amino acid exchanges reside in regions that were proposed by Bouche et al. (2002) as binding sites of neutralizing antibodies. Furthermore, this study provides evidence that the D7 H protein has an additional fifth
asparagine-linked oligosaccharide chain that is absent in the H of C2, D6 and vaccine viruses. This additional glycan might contribute to form or to mask epitopes of antibodies, as is known for the influenza A virus H (Skehel et al., 1984; Schulze, 1997; Hay et al., 2001) and reported for two of the four individual carbohydrate chains of the MV H protein from the Edmonston strain (Hu et al., 1994). These data imply the question of whether the D7 H has an altered antigenicity compared with C2, D6 and vaccine viruses.

The pattern of neutralizing epitopes on the H protein differs between the newly emerged D7 viruses, the previously circulating C2 and D6 viruses and the vaccine virus. The D7 H has lost at least two neutralizing epitopes that are present in the H of genotypes C2 and D6 and the vaccine virus. This has been demonstrated by the use of nine anti-H mAbs that were generated against an Edmonston-related MV and that bind to at least seven distinct domains on the H protein (Bouche et al., 2002). Binding studies with a recombinant H protein revealed that mAbs BH15 and BH125, which are unable to neutralize the D7 viruses, do not recognize the D7 H. In contrast, mAb BH97 neutralized D7 viruses more efficiently than C2 and D6 viruses and the vaccine virus, suggesting that the corresponding epitope on the D7 H has a better accessibility or higher binding affinity for this antibody. mAb BH47, identified to bind on the linear epitope at aa 244–250 on the H protein (Fournier et al., 1997), revealed that this neutralizing epitope is present on the H of C2 and D7 viruses and the vaccine virus, but is absent on the D6 H, where this mAb cannot bind. These data indicate that, besides the diversity in the pattern of neutralizing epitopes of the MV H protein, there are at least four, five and six neutralizing epitopes that are shared, respectively, by the vaccine virus and D7, D6 and C2 viruses. These results imply the question of whether the D7 H has an altered antigenicity compared with C2, D6 and vaccine viruses.

The altered pattern of neutralizing epitopes found in the D7 H protein did not influence the neutralizing capacity of polyclonal B-cell responses, as demonstrated in MV-infected cotton rats and humans. Like the cotton-rat sera, human sera from vaccinated, late-convalescent and early-convalescent individuals did not discriminate between D7 and other genotypes. Considering MVs of the genotypes C2, D6 and D7 and the vaccine strains, our data indicate that, although the pattern of neutralizing epitopes is different between genotypes, the neutralizing capacity of polyclonal B-cell responses is independent of genotype. This latter observation is in agreement with Klingele et al. (2000), who demonstrated that a given serum can have significantly different neutralization titres with respect to different MVs and that the more resistant viruses were not restricted to a certain clade or genotype. The apparently genotype-independent neutralizing capacity of polyclonal antibodies may be explained by the fact that there are at least three neutralizing epitopes on the H protein that are shared between all MVs of all genotypes included in our study and by the existence of a hierarchy of neutralizing epitopes. It has been reported that human serum antibodies have differing reactivity towards the different epitopes of a recombinant MV H protein (Ertl et al., 2003). A large proportion of human sera did not react with the domain occupied by mAb BH47, which showed a weak activity to block human serum antibodies. A few sera did not react with the binding domain of mAb BH15, which showed an intermediate blocking activity for human antibodies. In our study, mAb BH47 did not neutralize D6 viruses and mAb BH15 did not neutralize D7 viruses. For other mAbs, i.e. for BH6 recognizing the HNE, Ertl et al. (2003) reported a strong inhibition of serum antibodies. In our study, mAb BH6 neutralized all MVs investigated, independent of the genotype. Based on these data, we assume that a neutralizing epitope on the MV H protein that is conserved between MVs of different genotypes (e.g. the HNE) might be more relevant for binding of human antibodies than a non-conserved neutralizing epitope and might thus constitute an immunodominant epitope.

Our study documents a moderate degree of antigenic diversity of the MV H protein. The observation of the loss of two neutralizing epitopes on the D7 H compared with a vaccine virus implies the necessity to characterize the antigenicity of the H protein of newly emerged MVs.
However, the altered pattern of neutralizing epitopes on the D7 H did not affect the neutralizing capacity of polyclonal B-cell responses. Therefore, we assume that the rapid replacement of endemically circulating C2 and D6 genotypes by D7 was not mainly driven by immune selection through pre-existing neutralizing antibodies. The H glycoprotein is not only the prime target of neutralizing antibodies, but also represents an important determinant of MV cellular tropism (Stern et al., 1995; Johnston et al., 1999; Ohgimoto et al., 2001) and a factor that is critically involved in MV-induced immune suppression and pathogenesis (Niewiesk et al., 1997; Klagge et al., 2000). Therefore, it is tempting to speculate that the D7 H provides a selective advantage to D7 viruses by enhancing one of these capacities, rather than escaping from pre-existing neutralizing antibodies. As sera from vaccinated individuals also efficiently neutralized MVs of D7, we conclude that vaccination provides effective protection against this new genotype.

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