Depletion of measles virus glycoprotein-specific antibodies from human sera reveals genotype-specific neutralizing antibodies

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Measles virus (MV)-neutralizing antibodies in sera from vaccinated subjects are mainly directed against the haemagglutinin (H) protein. It has been shown previously that depletion of vaccination-induced H-specific antibodies by co-culture of sera with cells expressing the MV Edmonston strain H glycoprotein resulted in almost complete elimination of neutralizing activity. In the present study, MV H and/or fusion (F) protein-specific antibodies were depleted from sera of naturally immune subjects. Early convalescent samples were collected 1.5 years after a well-characterized measles outbreak in Luxembourg caused by a genotype C2 virus, whilst late convalescent samples were collected from healthy Dutch subjects born between 1960 and 1970. Depletion of both H- and F-specific antibodies completely eliminated virus-neutralizing (VN) activity against MV Edmonston. However, in the early convalescent samples, residual VN antibody against wild-type MV genotype C2 was detected. This demonstrated that, although the majority of MV-specific VN antibodies recognized epitopes conserved between different genotypes, genotype-specific VN epitopes were also induced. In sera depleted of H-specific antibodies only, VN activity against MV Edmonston was not completely eliminated, demonstrating the presence of F-specific VN antibodies. In conclusion, this study demonstrated that a fraction of VN antibodies induced by wild-type MV genotype C2 does not neutralize MV strain Edmonston. In addition, it was shown that, in sera from naturally immune donors, the majority of VN antibodies are specific for MV H protein, but up to 10% of neutralizing antibodies are specific for MV F protein.

INTRODUCTION

Measles remains an important cause of childhood morbidity and mortality in developing countries (WHO, 2008). The causative agent, measles virus (MV), is a member of the genus Morbillivirus, family Paramyxoviridae (Griffin, 2007). The virus is transmitted via the respiratory route and predominantly infects CD150-positive lymphocytes and dendritic cells (de Swart et al., 2007). MV infection is associated with immunosuppression, but paradoxically also results in life-long protection. Virus-neutralizing (VN) serum antibodies are the most important correlate of protection. VN antibody levels of 0.1–0.2 international units (IU) ml⁻¹, equivalent to titres of approximately 1:8 to 1:16, have been shown to protect from disease (Chen et al., 1990; Samb et al., 1995). VN antibodies are directed exclusively to the MV transmembrane glycoproteins, the haemagglutinin (H) and fusion (F) proteins, and mostly recognize conformational epitopes (Bouche et al., 2002).

We have demonstrated previously that, in sera collected from vaccinated subjects, MV-neutralizing antibodies are directed mainly to the H protein (de Swart et al., 2005). To this end, we used the stably transfected human melanoma cell line Mel-JuSo expressing the H or F protein derived from the MV Edmonston strain (de Swart et al., 1998). By incubating human serum on these cells for several days, MV glycoprotein-specific antibodies could specifically be depleted, allowing determination of their relative contribution to MV neutralization. Depletion of both H- and F-specific antibodies in all cases resulted in complete elimination of detectable VN activity, demonstrating the technical feasibility of this approach. Depletion of F-specific antibodies resulted in only a minor reduction in VN activity, whilst depletion of H-specific antibodies in the majority of the samples completely eliminated VN activity, although in a minor percentage a low VN titre remained (de Swart et al., 2005).

MV-specific antibody levels induced by natural infection are higher than those induced by vaccination (van den Hof et al., 1999; Itoh et al., 2002). Primary and secondary vaccination failures have been associated with susceptibility
to subclinical or clinical wild-type (wt) MV infection (Reyes et al., 1987; Fine & Zell, 1994; De Serres et al., 1995; Sutcliffe & Rea, 1996; Paunio et al., 2000), whereas naturally immune subjects only rarely become susceptible to subclinical MV reinfection (Muller et al., 1996). It has been speculated that susceptibility to reinfection could be facilitated by genetic differences between the MV vaccine virus and currently circulating wt MV strains (Tamin et al., 1994; Santibanez et al., 2005). Although MV is a monotypic virus, genetic differences have allowed identification of eight clades and 23 genotypes, with the majority of genetic differences in the 3′ end of the nucleoprotein gene and in the complete H gene (Rota et al., 2009). All live-attenuated MV vaccine strains are members of clade A and are genetically virtually identical to MV Edmonston isolated in the USA in 1954 (Enders & Peebles, 1954). Circulation of current wt MV strains in populations with high vaccination coverage could theoretically result in the selection of escape mutants (Kühne et al., 2006). However, the existence of MV strains that escape vaccine-induced immunity has not been demonstrated (Santibanez et al., 2005), most likely due to structural constraints limiting the possibilities of the virus to alter its surface glycoproteins (Frank & Bush, 2007).

Here, we have used the antibody depletion approach to study the relative contribution of H- and F-specific antibodies in sera from naturally immune subjects to VN activity against both MV Edmonston and wt MV. We used sera collected approximately 1.5 years after a measles outbreak in Luxembourg in 1996 (Hanses et al., 2000). A wt MV strain associated with this outbreak (MVi/Reuler.LUX/17.96) was isolated from peripheral blood mononuclear cells collected from one of the patients during the acute phase of the disease, and was shown to be a member of genotype C2 (Hanses et al., 2000). Antibodies were depleted from the sera using cells expressing the genotype A MV glycoproteins H or F, and VN activity was subsequently assessed against MV genotypes A and C2. In addition, a second cohort of late convalescent (30–40 years after measles) Dutch sera was also used for antibody depletion. As the genotype that was associated with natural infection of these subjects was unknown, these sera were only tested for their neutralizing activity against genotype A MV Edmonston.

**METHODS**

**Serum samples.** Early convalescent sera (n=30) were collected in 1997 approximately 1.5 years (median 551 days, range 461–603) after acute infection with wt MV genotype C2 in Luxembourg (Hanses et al., 2000). For 22 of the subjects, it had been documented that they were unvaccinated against MV. For the remaining subjects, vaccination status was unknown, but they were also assumed to be unvaccinated. All subjects had developed the full spectrum of measles-associated clinical symptoms, but recovered uneventfully. Late convalescent samples were collected from the serum bank of the Erasmus MC Department of Virology. Sera were selected from 32 healthy subjects born in the period 1960–1970, i.e. before the introduction of MV vaccination in The Netherlands in 1976.

Therefore, these subjects most likely experienced natural measles during childhood, i.e. approximately 30–40 years before sample collection. All sera were heat-inactivated (30 min, 56 °C) before use. The sera were subsequently diluted 1:10 in RPMI 1640 supplemented with penicillin (100 U ml⁻¹), streptomycin (100 µg ml⁻¹) and L-glutamine (2 mM) for use in depletion studies.

**Cells and media.** The adherent cell line Mel-JuSo/wt (Johnson et al., 1981), transfected or not with the Edmonston H (Mel-JuSo/MV-H) or F (Mel-JuSo/MV-F) gene (de Swart et al., 1998), was cultured in RPMI 1640 supplemented with antibiotics and 10% heat-inactivated fetal bovine serum (FBS). In antibody-depletion assays, the cells were seeded in 96-well flat-bottomed plates. When monolayers were approximately 90% confluent, the medium was replaced by human serum diluted 1:10 in RPMI 1640 and cells were cultured for 4 days at 37 °C. Subsequently, supernatants were collected and incubated for another 4 days on a second plate with subconfluent Mel-JuSo cell monolayers. By selection of the appropriate cell lines, this procedure resulted in depletion of H- and/or F-specific antibodies (see Fig. 1a; de Swart et al., 2005).

**Viruses and neutralization assays.** A plaque-purified variant of the Edmonston B virus (van Binnendijk et al., 1989) was grown in Vero cells in RPMI 1640 supplemented with antibiotics and 10% FBS. Wt MV strain MVi/Reuler.LUX/17.96 (GenBank accession no. AJ244041, genotype C2) was isolated from peripheral blood mononuclear cells collected 3 days after the onset of rash by co-cultivation with human Epstein–Barr virus-transformed B-lymphoblastic cells, as described previously for other wt MV strains (El Mubarak et al., 2006). For the present study, a virus stock was grown in Vero cells transfected with CD150 (cell line kindly provided by Dr Y. Yanagi; Ono et al., 2001). Virus-neutralizing antibody titres were determined by end-point titration as described previously (de Swart et al., 2005). Briefly, a twofold dilution series of the culture supernatants was tested for the capacity to neutralize 100 50% tissue culture infectious doses of MV Edmonston B or MVi/Reuler.LUX/17.96 on Vero or Vero–CD150 cells, respectively. In each experiment, sera were tested in triplicate and data are shown as the geometric mean titres of two independent experiments. For MV Edmonston B, the second international standard serum for measles (5 IU ml⁻¹, serum 66/202; WHO International Laboratory for Biological Standards, NIBSC, UK) was included in the assay to allow expression of the results as IU ml⁻¹.

**Detection of H- and F-specific IgG antibody levels.** H- and F-specific IgG antibody levels were determined by flow cytometry, as described previously (de Swart et al., 1998, 2005). Briefly, Mel-JuSo/MV-H or Mel-JuSo/MV-F cells were incubated with culture supernatant diluted 1:10 in PBS supplemented with 10% FBS (i.e. final test serum dilution of 1:100). After incubation for 1 h on ice, the cells were washed and incubated with a fluorescein isothiocyanate-conjugated goat anti-human IgG polyclonal antibody [F(ab')² fragments; Dako]. After a further 1 h incubation on ice, cells were washed again and fluorescence was measured on a FACSCalibur (Becton Dickinson). Data were obtained by determining the histogram peak channel for each sample and expressed as arbitrary fluorescence units (AFU).

**RESULTS**

**Depletion of H- and/or F-specific antibodies**

Human sera collected from early or late convalescent naturally immune donors were incubated for two subsequent periods of 4 days on transfected human melanoma cells expressing the MV transmembrane glycoproteins H or

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As shown in Fig. 1(a), the study design included four different incubation conditions, resulting in no depletion (condition 1), depletion of H-specific (condition 2) or F-specific (condition 3) antibodies only, or depletion of both H- and F-specific antibodies (condition 4). The four resulting culture supernatants and the original materials (condition 0) were used to measure MV H- and F-specific IgG antibodies (Figs 2 and 3, respectively). As shown in Figs 2a and 3a, early convalescent sera contained higher H- and F-specific antibody levels than late convalescent sera (grey symbols). Incubation of the sera on Mel-JuSo/MV-H cells resulted in specific depletion of H-specific IgG antibodies (Fig. 2b, d), whilst F-specific IgG antibody levels remained unaffected (Fig. 2c). In contrast, incubation of the sera on Mel-JuSo/MV-F cells resulted in specific depletion of F-specific IgG antibodies (Fig. 3c, d), whilst H-specific IgG antibody levels remained unaffected (Fig. 3b). In both cases, antibody depletion in the sera with the highest antibody levels in some cases seemed incomplete.

Effect of antibody depletion on VN activity against MV genotype A

The transfected cell lines used for antibody depletion expressed the H or F genes of MV Edmonston. In a previous study, we depleted MV-specific antibodies from sera collected from vaccinated subjects (de Swart et al., 2005), i.e. the immunizing and depleting antigens were completely homologous (Fig. 1b, shown in red). However, in sera collected from naturally immune subjects as tested in the present study, MV-specific antibodies were induced by a wt MV strain, which normally belongs to a different genotype from MV Edmonston. The early convalescent sera used here were from subjects infected with a genotype C2 virus. Antibody depletion using cells expressing the Edmonston H or F protein therefore only depleted antibodies that cross-reacted between genotypes A and C2, but could not have resulted in the depletion of genotype C2-specific antibodies (Fig. 1b, shown in red and green).

Fig. 1. (a) Study design. Human sera collected early (1.5 years, n=30) or late (30–40 years, n=32) after wt MV infection were diluted 1 : 10 in RPMI 1640 (condition 0) to allow two 4-day co-cultures with Mel-JuSo cells expressing MV H or F protein. This resulted in four conditions: condition 1, no depletion (culture on Mel-JuSo/wt only); condition 2, depletion of H-specific antibodies (culture on Mel-JuSo/MV-H only); condition 3, depletion of F-specific antibodies (culture on Mel-JuSo/MV-F only); condition 4, depletion of both H- and F-specific antibodies (culture on both Mel-JuSo/MV-H and Mel-JuSo/MV-F). (b) Illustration of the depletion of MV-specific antibodies binding to all MV genotypes (shown in red) or genotype-specific antibodies (blue for genotype A, green for genotype C2).
Fig. 2. MV H-specific IgG levels, measured by immunofluorescence using FACS on Mel-JuSo/MV-H expressing cells and expressed in arbitrary fluorescence units (AFU). Filled symbols represent early convalescent samples and shaded symbols represent late convalescent samples. Depletion conditions (Fig. 1a) are: no depletion (a), H depletion (b), F depletion (c) and F and H depletion (d).

Fig. 3. MV F-specific IgG levels, measured by immunofluorescence using FACS on Mel-JuSo/MV-F-expressing cells and expressed in arbitrary fluorescence units (AFU). Filled symbols represent early convalescent samples and shaded symbols represent late convalescent samples. See Figs 1a and 2 for depletion conditions.
When depleting both MV H- and F-specific antibodies from sera collected from naturally immune subjects, VN activity against MV Edmonston should be completely eliminated. The genotype C2-specific antibodies that remain after depletion cannot recognize this genotype A virus (Fig. 1b, antibodies shown in green). Indeed, depletion of both H- and F-specific antibodies resulted in the complete loss of detectable VN activity against MV Edmonston in both early and late convalescent sera (Fig. 4d). Apparently, the seemingly incomplete antibody depletion in a number of the sera with high antibody levels, as shown in Figs 2 and 3, did not result in residual neutralizing activity. As shown in Fig. 4b, c, depletion of H-specific antibodies resulted in a stronger reduction in VN antibody levels than depletion of F-specific antibodies. However, especially in sera with VN antibody levels above 3 IU ml\(^{-1}\), approximately 10% of the original VN activity remained after depletion of H-specific antibodies.

**Effect of antibody depletion on VN activity against wt MV genotype C2**

The early convalescent samples used for measurement of VN antibody levels against MV Edmonston (Fig. 4, filled symbols) were also used for detection of neutralizing activity against MVi/Reuler.LUX/17.96 (genotype C2). A number of the samples from which MV Edmonston H- and F-specific antibodies had been depleted and that showed no residual VN activity against MV Edmonston (Fig. 4d) still showed neutralizing activity against wt MV genotype C2 (Fig. 5d). This was especially true for the sera with the highest initial VN antibody titre (above 2000). Again, depletion of H-specific antibodies resulted in a stronger reduction in VN activity than depletion of F-specific antibodies (Fig. 5b, c, respectively), but the reduction in VN activity against the wt strain was not as strong as that against MV Edmonston (compare Figs 4b and 5b).

**DISCUSSION**

We depleted MV H- and/or F-specific antibodies from the sera of naturally immune donors using melanoma cells expressing the H or F genes derived from the Edmonston strain. Depletion of antibodies of both specificities resulted in complete elimination of neutralizing activity against MV Edmonston, but in sera with high specific antibody levels, a substantial part of the neutralizing activity against the genotype C2 wt MV strain that had induced the antibodies remained, demonstrating that a subset of the VN antibodies recognized VN epitopes present on C2 but absent from genotype A viruses. Although the study confirmed our earlier observations that the majority of VN antibodies are directed against the H protein,
approximately 10% of VN activity against MV Edmonston remained after depletion of H-specific antibodies, demonstrating the presence of F-specific VN antibodies.

After depletion of MV Edmonston H- and F-specific antibodies from sera collected 1.5 years after a genotype C2-associated measles outbreak in Luxembourg, we could still detect VN activity against the homologous genotype C2 wt MV strain. However, these 'genotype-specific' VN antibodies (which could not neutralize MV Edmonston) in all cases constituted less than half of the total level of VN antibodies: the majority of the VN antibodies could recognize both MV genotypes. This is in accordance with the fact that true escape mutants for MV have never been detected (Bouche et al., 2002; Santibanez et al., 2005).

We demonstrated previously that depletion of H-specific antibodies from the sera of vaccinated subjects resulted in an almost complete elimination of VN activity against MV strain Edmonston (de Swart et al., 2005). However, VN epitopes have also been described in the F protein (Wiesmüller et al., 1992; Atabani et al., 1997; Fayolle et al., 1999). In the present study, we used sera collected from naturally immune instead of vaccinated subjects. As expected, MV glycoprotein-specific IgG levels and MV Edmonston-specific VN antibody levels were, on average, higher than those in vaccinated subjects measured in the previous study (van den Hof et al., 1999; Itoh et al., 2002). The contribution of F-specific antibodies to virus neutralization was mainly observed in the sera with relatively high titres, suggesting that this observation was mainly due to the overall higher specific antibody levels in the naturally immune subjects. Indeed, in our previous study, there were also a few samples with relatively high titres in which some VN activity remained after depletion of H-specific antibodies (de Swart et al., 2005). F-specific VN antibodies seemed to cluster on a straight line parallel with the diagonal at values approximately tenfold lower than the antibody levels detected in the untreated sera (Fig. 4b), suggesting that F-specific VN antibodies constituted approximately 10% of the total neutralizing antibody pool.

The antibody depletion approach using living cells expressing a viral transmembrane glycoprotein in its native conformation again proved a feasible method to study the role of protein-specific antibody responses in virus neutralization. A similar approach was used by Sastre et al. (2005) who depleted respiratory syncytial virus (RSV) glycoprotein-specific antibodies from polyclonal sera by incubation with cells infected with recombinant vaccinia virus expressing the respective RSV proteins. For MV, adsorption of H-specific antibodies to acetone-treated MV-infected cells has been used to characterize the role of MV F-specific antibodies (Armstrong et al., 1979; Sato et al., 1989). However, in the present study, we depleted specific antibodies by using cells expressing the MV glycoproteins in their native conformation.
In conclusion, we have further characterized the relative contribution of MV H- and F-specific antibodies to virus neutralization. In naturally immune subjects, approximately 10% of VN antibodies were directed to the MV F protein, but the current study confirms our previous conclusion that the majority of VN antibodies are directed to MV H protein. Furthermore, the present study demonstrates that infection with wt MV may induce both cross-specific and genotype-specific VN antibodies, but the cross-specific antibodies constitute the majority of the total VN activity, and thus the appearance of mutant virus strains that can escape vaccine-induced immunity seems unlikely.

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