Cell tropism of wild-type measles virus is affected by amino acid substitutions in the P, V and M proteins, or by a truncation in the C protein

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Two nucleotide differences in the P/C/V and M genes between B95a cell- and Vero cell-isolated wild-type measles viruses (MV) have previously been found from the same patient. The nucleotide difference in the P/C/V gene resulted in an amino acid difference (M175I) in the P and V proteins and a 19 aa deletion in the C protein. The nucleotide difference in the M gene resulted in an amino acid difference (P64H) in the M protein. To verify this result and to examine further whether the amino acid difference or truncation is important for MV cell tropism, recombinant MV strains containing one of the two nucleotide substitutions, or both, were generated. It was found that the P64H substitution in the M protein was important for efficient virus growth and dissemination in Vero cells and that the M175I substitution in the P and V protein or truncation of the C protein was required for optimal growth.

The measles virus (MV) RNA genome encodes six structural proteins: nucleocapsid (N) protein, phospho (P) protein, matrix (M) protein, fusion (F) protein, haemagglutinin (H) protein and large polymerase (L) protein. The P and L proteins form the RNA-dependent RNA polymerase and both proteins associate with the nucleocapsid to form the ribonucleoprotein (RNP) complex. The P and N proteins form a complex that is used for the encapsidation of RNA during replication. The M protein lines the inner surface of the host-cell plasma membrane and is involved in virus assembly by targeting viral envelope proteins and the RNP to the site of virus budding. The H and F proteins are envelope proteins responsible for receptor binding and mediating membrane fusion, respectively (reviewed by Griffin, 2001; Horikami & Moyer, 1995). In addition to the six structural proteins, the P cistron encodes the C, V and R proteins, whose functions remain to be elucidated. Several reports have indicated that the non-structural C and V proteins are related to the pathogenicity of MV (Mrkic et al., 2000; Patterson et al., 2000; Tober et al., 1998; Valsamakis et al., 1998). Recently, it has been shown that the C and V proteins of MV have anti-interferon activity (Palosaari et al., 2003; Shaffer et al., 2003; Takeuchi et al., 2003a; Yokota et al., 2003).

Wild-type MV can be efficiently isolated in B95a cells (Kobune et al., 1990). In this report, we have used the term ‘wild-type’ for MV strains that have been isolated and passaged exclusively in B95a cells or human B-lymphoid cells. Interestingly, wild-type MV strains isolated in B95a cells have phenotypes distinct from those isolated or adapted in Vero cells. Wild-type MV strains isolated in B95a cells induce clinical signs resembling those of human measles in experimentally infected cynomolgus and squirrel monkeys, whereas MV strains isolated or adapted to Vero cells do not induce such clinical signs in monkeys (Kobune et al., 1990, 1996). In addition, wild-type MV strains replicate efficiently in B95a cells but poorly in Vero cells, whereas MV strains isolated or adapted in Vero cells replicate efficiently in both B95a and Vero cells (Kobune et al., 1990). To date, the difference in cell tropism of wild-type and laboratory strains of MV has been explained by their receptor usage. Wild-type strains of MV use signalling lymphocyte activation molecule (SLAM, or CD150) as a cellular receptor (Erlenhoefer et al., 2001; Hsu et al., 2001; Tatsuo et al., 2000). In contrast, laboratory strains of MV, such as the Edmonston strain, use CD46 (Dörg et al., 1993; Naniche et al., 1993) and SLAM as cellular receptors. However, the cell tropism of MV is still controversial (Oldstone et al., 2002).

In order to investigate the molecular mechanisms underlying the phenotypic differences between B95a cell-isolated and Vero cell-isolated MV strains, we determined the
nucleotide sequence of the complete genomes of a B95a cell-isolated strain (IC-B) and a Vero cell-isolated strain (IC-V) from the same patient (Kobune et al., 1990). We found only two nucleotide differences between the IC-B and IC-V strains: one in the P/C/V gene at nt 2331 (G→A) and the other in the M gene at nt 3628 (C→A). These nucleotide differences predicted amino acid differences in the P, V and M proteins, as well as a 19 aa deletion in the C protein of the IC-V strain (Takeuchi et al., 2000). No differences were found between the two H genes.

In order to verify this result and to examine further which nucleotide difference is important for these phenotypes, recombinant viruses containing one of the two nucleotide substitutions, or both, were generated. IC(P/M) virus corresponding to the IC-B strain of wild-type MV (Kobune et al., 1990) was recovered from plasmid p(+)MV(P/M) as reported previously (Takeda et al., 2000). In this paper, we refer to the IC323 virus and p(+)MV323 plasmid (Takeda et al., 2000) as IC(P/M) virus and p(+)MV(P/M) plasmid, respectively, to indicate clearly the introduced mutation(s). Three additional plasmids, p(+)MV(mP/M) carrying the nt 2331 G→A substitution in the P/C/V gene, p(+)MV(P/mM) carrying the nt 3628 C→A substitution in the M gene and p(+)MV(mP/mM) carrying both nucleotide substitutions, were constructed from p(+)MV(P/M) by exchanging a cDNA fragment of the IC-V strain or by introducing mutated fragments synthesized by PCR. Infectious MV strains were recovered by the method previously reported (Radecke et al., 1995; Takeda et al., 2000).

In B95a cells, IC(P/M), IC(mP/M), IC(P/mM) and IC(mP/mM) replicated efficiently and there was no significant difference in replication kinetics among the rescued viruses (Fig. 1a). However, IC(P/M) (Fig. 2a) and IC(mP/M) (Fig. 2b) induced large syncytia in B95a cells similar to those of the IC-B strain, whereas IC(P/mM) (Fig. 2c) and IC(mP/mM) (Fig. 2d) induced small syncytia similar to those of the IC-V strain.

In Vero cells, the IC-B strain corresponding to IC(P/M) replicated at a very low level, as has been reported previously (Takeda et al., 2000; Takeuchi et al., 2002) and IC(mP/M) also replicated poorly (Fig. 1b). In contrast, IC(mP/mM) replicated efficiently in Vero cells (Fig. 1b). Since IC(mP/mM) corresponds to the IC-V strain, this result was in good agreement with the previous observation that the IC-V strain replicates efficiently in Vero cells (Kobune et al., 1990). IC(P/mM) replicated efficiently in Vero cells, but less so than IC(mP/mM) (Fig. 1b). IC(P/M) and IC(mP/M) did not induce apparent cytopathic effects in Vero cells over a period of 6 days (Fig. 2e and f). IC(mP/mM) induced the formation of rounded cells (Fig. 2h), but did not induce typical syncytia. IC(P/mM) also induced the formation of rounded cells, but this phenotype was much less pronounced than with IC(mP/mM) (Fig. 2g). To detect MV protein synthesis in Vero cell culture, immunofluorescent analysis was performed 6 days post-infection. No, or very few, positive cells were detected among IC(P/M)-infected (Fig. 2i) and IC(mP/M)-infected (Fig. 2j) Vero cells under our experimental conditions. Several groups of positive cells were detected in IC(P/mM)-infected Vero cells (Fig. 2k) and almost all IC(mP/mM)-infected Vero cells were positive (Fig. 2l).

To study the entry and dissemination of recombinant MV strains in Vero cells in more detail, we introduced the enhanced GFP (EGFP) gene into the recombinant viruses with the nucleotide substitutions in the P/C/V and/or M genes. The plasmid p(+)MV(P/M)-EGFP carrying the full-genome cDNA of the pathogenic IC-B strain and the EGFP gene has been described previously (Hashimoto et al., 2002). In this paper, we refer to plasmid p(+)MV323-EGFP and IC323-EGFP as p(+)MV(P/M)-EGFP and IC(P/M)-EGFP, respectively, to indicate clearly the introduced mutation(s).

To make recombinant viruses expressing EGFP and carrying one of the two nucleotide substitutions, or both, three
plasmids, p(+)-MV(M)-EGFP, p(+)-MV(P/M)-EGFP and p(+)-MV(mM)-EGFP were constructed from p(+)-MV(P/M)-EGFP by exchanging corresponding fragments of p(+)-MV(M), p(+)-MV(P/M) or p(+)-MV(mM), respectively. Infectious MV strains were recovered as reported previously (Radecke et al., 1995; Takeda et al., 2000). When Vero cells were infected with the four recombinant MV strains expressing EGFP, entry and dissemination of MV were easily monitored by the expression of EGFP. IC(P/M)-EGFP barely disseminated in the culture (Fig. 3a, e and i). Similarly, IC(mP/M)-EGFP did not disseminate efficiently (Fig. 3b, f and j). Interestingly, a spread of IC(mP/M)-EGFP to bystander cells, possibly by microfusion at sites of cell-to-cell contact, was observed 6 days post-infection (Fig. 3j). In sharp contrast, IC(mP/mM)-EGFP efficiently disseminated in the culture (Fig. 3d and h) and almost all cells were infected by 6 days post-infection (Fig. 3l). IC(P/mM)-EGFP also disseminated in the culture, but to a lesser extent than IC(mP/mM)-EGFP (Fig. 3c, g and k).

Fig. 2. Cytopathic effects of recombinant viruses in B95a and Vero cells. B95a cells were infected with IC(P/M) (a), IC(mP/M) (b), IC(P/mM) (c) and IC(mP/mM) (d) and cells were photographed under a microscope 1 day post-infection. Small syncytia induced by IC(P/mM) and IC(mP/mM) are indicated by arrows. Vero cells were infected with IC(P/M) (e), IC(mP/M) (f), IC(P/mM) (g) and IC(mP/mM) (h) and cells were photographed 6 days post-infection. Vero cells were infected with IC(P/M) (i), IC(mP/M) (j), IC(P/mM) (k) and IC(mP/mM) (l), and infected cells were detected by immunofluorescent analysis using a monoclonal antibody against the H protein (B5) (Sato et al., 1985) and FITC-conjugated anti-mouse IgG (i–l). Magnification ×5 (a–h); ×10 (i–l).
To investigate whether the difference in cell tropism of IC(P/M) and IC(mP/mM) is specific for Vero cells or not, various cell lines were infected with IC(P/M) and IC(mP/mM). IC(mP/mM) produced a viral titre that was more than 10 times higher than that of IC(P/M) in Raji and MRC-5 cells. Limited growth of IC(mP/mM), but not IC(P/M), was detected in HeLa, A549, 293T and Chinese hamster ovary cells. In Jurkat and Madin–Darby canine kidney cells, growth was not observed for either virus (data not shown).

What are the possible mechanistic effects of these point substitutions in the P, V and M proteins and the truncation in the C protein? Because the M proteins of negative-strand RNA viruses are known to act as central players in virus assembly (reviewed by Lamb & Kolakofsky, 2001), the P64H substitution in the M protein may affect virus assembly.

Interestingly, P64 is conserved in some morbilliviruses, Rinderpest virus and peste-des-petits-ruminants virus and may have an important role. Since IC(P/mM)-EGFP and IC(mP/mM)-EGFP efficiently disseminated in Vero cells (Fig. 3), MV containing the M protein with the P64H substitution may bud from Vero cells more efficiently and/or infect Vero cells more successfully. We are currently measuring the budding and infection efficiency of MV using strains expressing EGFP. An electron microscopic examination could not detect significant differences in the shape of the virus particles of IC(P/M) and IC(mP/mM) (data not shown). In addition to the P64H substitution in the M protein, the substitution in the P or V protein (M175I) or the 19 aa truncation of the C protein was required for optimal MV growth in Vero cells (Fig. 1) and dissemination in Vero cells (Fig. 3). Because the P protein is one of the

Fig. 3. Dissemination of recombinant viruses expressing EGFP in Vero cells. Vero cells were infected with IC(P/M)-EGFP (a, e and i), IC(mP/M)-EGFP (b, f and j), IC(P/mM)-EGFP (c, g and k) or IC(mP/mM)-EGFP (d, h and l). Expression of EGFP was monitored by fluorescent microscopy 2 (a–d), 4 (e–h) or 6 (i–l) days post-infection. Magnification × 5.
components of viral RNA polymerase complexes, MV RNA synthesis may be up-regulated by the point amino acid substitution (M175I) in the P protein, as has been predicted previously (Takeda et al., 1998). Since a mutation in the C protein of MV up-regulates viral mRNA synthesis (Reutter et al., 2001), the 19 aa truncation of the C protein may also up-regulate viral RNA synthesis. On the other hand, recombinant MV strains deficient for expression of the C or V protein grew normally in Vero cells (Radecke & Billeter, 1996; Schneider et al., 1997), suggesting that the contribution of the C or V protein is less likely in our case. At present, we cannot distinguish the effect of the amino acid point substitution (M175I) in the P and V proteins from that of the 19 aa truncation of the C protein, since it is impossible to introduce a mutation to one protein without changing the amino acid sequence of other proteins. Evaluation of the effect of the point substitution (M175I) in the P and V proteins and the truncation of the C protein on RNA synthesis is in progress using a MV CAT minireplicon assay. It is of interest to examine the presence of amino acid substitutions in the P, G, V or M proteins in other original and Vero cell-adapted MV strain pairs (Kouomou & Wild, 2002; Manchester et al., 2000; Nielsen et al., 2001; Takeda et al., 1998). One of the most critical questions is how MV strains with wild-type H protein can infect SLAM-negative Vero cells. One possibility is that those MV strains recognize CD46 on Vero cells at a low efficiency, which would allow virus entry into the cells (Kouomou & Wild, 2002; Manchester et al., 2000; Massé et al., 2002; Schneider et al., 2002; Vongpunawat et al., 2004). Alternatively, those MV strains may infect cells using an as-yet-undefined third MV receptor (Andres et al., 2003; Hashimoto et al., 2002; Manchester et al., 2002; McQuaid & Cosby, 2002; Takeuchi et al., 2002, 2003b). A monoclonal antibody against CD46 did not block the infection of IC(mP/mM)-EGFP to Vero cells (data not shown), favouring the second possibility.

It should be noted that MV strains with an expanded cell tropism, such as the IC-V strain, are pathogenic for monkeys, while MV strains with a narrow cell tropism, such as the IC-B strain, are pathogenic for monkeys (Kobune et al., 1990). At present, we do not know which nucleotide difference is responsible for pathogenicity in monkeys. Experimental infection of monkeys with IC(mP/M) and IC(P/mM) is required to elucidate this point.

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