Measles Viruses Possessing the Polymerase Protein Genes of the Edmonston Vaccine Strain Exhibit Attenuated Gene Expression and Growth in Cultured Cells and SLAM Knock-In Mice

Makoto Takeda, Shinji Ohno, Maino Tahara, Hiroki Takeuchi, Yuta Shirogane, Hirofumi Ohmura, Takafumi Nakamura, and Yusuke Yanagi

Department of Virology, Faculty of Medicine, Kyushu University, Fukuoka 812-8582, Japan; Core Facility for Therapeutic Vectors, The Institute of Medical Science, The University of Tokyo, 4-6-1, Shirokanedai Minato-ku, Tokyo 108-8639, Japan; and RNA and Biofunctions, PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

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Live attenuated vaccines against measles have been developed through adaptation of clinical isolates of measles virus (MV) in various cultured cells. Analyses using recombinant MVs with chimeric genomes between wild-type and Edmonston vaccine strains indicated that viruses possessing the polymerase protein genes of the Edmonston strain exhibited attenuated viral gene expression and growth in cultured cells as well as in mice expressing an MV receptor, signaling lymphocyte activation molecule, regardless of whether the virus genome had the wild-type or vaccine-type promoter sequence. These data demonstrate that the polymerase protein genes of the Edmonston strain contribute to its attenuated phenotype.

Measles is a highly contagious disease associated with high morbidity and mortality. Measles virus (MV), the causative agent of the disease, is an enveloped virus with a nonsegmented negative-strand RNA genome of ~16 kb that is classified in the genus Morbillivirus in the family Paramyxoviridae (12). The genome is encapsidated by the nucleocapsid (N) protein and associated with a viral RNA-dependent RNA polymerase composed of two subunits, phosphoprotein (P protein) and large (L) protein. The genome and these proteins form a helical ribonucleoprotein complex that acts as the active template for transcription and replication (12). The incidence and mortality of measles have been remarkably reduced in many countries as the vaccine coverage rate increases (12).

Live attenuated MV vaccines have been generated via many rounds of passage of clinical isolates of MV in various cultured cells (12, 29). The Edmonston strain, isolated in 1954 (9), was used as a seed strain to generate live attenuated vaccines (17, 29). Although some of the molecular mechanisms by which Edmonston lineage vaccines have adapted to grow in various cultured cells have been elucidated (32, 33, 40), the mechanisms of their attenuation are poorly understood. In the present study, we generated various recombinant MVs with chimeric genomes of the Edmonston vaccine and wild-type strains of MV, and we found that viruses possessing the polymerase protein genes of the Edmonston strain exhibited attenuated MV gene expression and growth in cultured cells as well as in mice expressing a cellular receptor for MV, human signaling lymphocyte activation molecule (hSLAM) (21). These data provide a molecular basis for the avirulence of the Edmonston strain in vivo.

SLAM is a universal receptor for all MV strains, while CD46 functions as an additional receptor for vaccine strains but not for wild-type strains (41, 43). Thus, both wild-type and Edmonston strains replicate efficiently and produce high titers of progeny viruses in SLAM-expressing cells (10, 14). However, when plaque assays were performed, the Edmonston lineage strains produced smaller plaques than wild-type strains of MV (Fig. 1A). Wild-type strains of MV (JPN/50.98/1 and JPN/26.99/1) were isolated from patients with measles and passaged two or three times in B95a cells (15) to make stocks for experiments (kind gifts from H. Sakata). The Edmonston strain obtained from the American Type Culture Collection (ATCC) is designated the Ed-ATCC strain in this paper. Recombinant Edmonston tag (Ed-tag) (26) and wild-type IC323 (39) MV clones were recovered from cDNAs and grown in Vero/hSLAM cells (23). To clarify the genes responsible for the small-plaque-forming phenotype of the Edmonston strain, six recombinant MVs that each contained one of the Ed-tag strain genes in the backbone of the genome of the wild-type IC323 strain expressing enhanced green fluorescent protein (IC323-EGFP) (13) were prepared and designated IC/Ed-N-EGFP, IC/Ed-P-EGFP, IC/Ed-M-EGFP, IC/Ed-F-EGFP, IC/Ed-H-EGFP, and IC/Ed-L-EGFP. IC/Ed-N-EGFP, IC/Ed-P-EGFP, IC/Ed-M-EGFP, IC/Ed-F-EGFP, IC/Ed-H-EGFP, and IC/Ed-L-EGFP were reported previously and referred to as m2, m3, m7, m8, and m9, respectively (34). When the recombinant MVs were used to infect Vero/hSLAM cells, IC/Ed-P-EGFP and IC/Ed-L-EGFP produced smaller plaques than wild-type IC323-EGFP (Fig. 1B). These data indicate that the P and L genes of the Ed-tag strain contribute to the decreased plaque sizes produced by the Ed-tag strain. In addition to the P protein, the P gene encodes two accessory gene products, V and C (12). The V protein directly interferes with host interferon (IFN) induction and IFN signaling pathways, while the C protein modulates viral RNA synthesis to circumvent IFN induction (19). It should be noted that, unlike the V proteins of the Ed-ATCC and wild-

* Corresponding author. Mailing address: Department of Virology, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Phone: 81-92-642-6138. Fax: 81-92-642-6140. E-mail: mtakeda@virology.med.kyushu-u.ac.jp.

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type MV strains, the V protein of the Ed-tag strain is defective in counteracting IFN signaling pathways owing to tyrosine-to-histidine and cysteine-to-arginine substitutions at amino acid positions 110 and 272 (Y110H and C272R), respectively (5, 8, 11, 22). Substitutions at these positions are not unique to the Ed-tag strain and have been found in other vaccines and cultured cell-adapted MV strains, including the CAM-70 vaccine (4), the Changchun-47 vaccine (4), the chicken embryo fibroblast-adapted D-CEF strain (1), and the Vero cell-adapted 94YTV strain (containing the C272R substitution; our unpublished observation). These findings prompted us to carry out further experiments using the P gene of the Ed-tag strain.

To quantify the expression levels of MV genes, four recombinant MVs expressing Renilla luciferase instead of EGFP were generated. The wild-type IC323 strain expressing Renilla luciferase (IC323-Luci) was reported previously (38). The P and/or L genes of the Ed-tag strain were introduced into the IC323-Luci genome, thereby generating IC/Ed-P-Luci, IC/Ed-L-Luci, and IC/Ed-PL-Luci. The Renilla luciferase activities induced by these recombinant MVs were analyzed in various cell types expressing SLAM (Vero/hSLAM [23], CV1/hSLAM, HeLa/hSLAM, A549/hSLAM [36], CHO/hSLAM [41], B95a, MT2, Raji, Ramos, BJAB-B95-8, and C91/PL) or an unidentified MV receptor on epithelial cells (NCI-H358) (38). CV-1/ hSLAM and HeLa/hSLAM cells were generated by cotransfecting CV1 and HeLa cells with the eukaryotic expression vector pCA7 (36), a derivative of pCAGGS (20) encoding human SLAM, and the selection vector pCXN2 (20), encoding a neomycin resistance gene, followed by selection in the presence of Geneticin (G418; Nacalai Tesque). Among all these cell lines, IC/Ed-P-Luci and IC/Ed-L-Luci showed reduced Renilla luciferase expression levels compared with wild-type IC323-Luci (Fig. 2A and data not shown). The luciferase activities induced by IC/Ed-PL-Luci were even lower than those induced by IC/Ed-P-Luci and IC/Ed-L-Luci among all the cell lines examined (Fig. 2A and data not shown). These data indicate that introduction of the P and L genes of the Ed-tag strain into recombinant virus genomes reduces viral gene expression and growth, regardless of the cell type. The viral mRNA levels in Vero/hSLAM cells infected with the recombinant MVs were also quantified. Confluent monolayers of Vero/hSLAM cells in 6-cm culture plates were infected with 1.0 × 10^4 PFU of IC323-Luci, IC/Ed-P-Luci, IC/Ed-L-Luci, or IC/Ed-PL-Luci. Multiple rounds of infection were blocked by a fusion-blocking peptide (Peptide Institute) (28). At 18 h postinfection (p.i.), mRNAs were purified from the cells using TRIzol reagent (Invitrogen) and an Oligotex-dT30 mRNA purification kit (TaKaRa Bio Inc.), reverse transcribed into cDNAs with an iScript cDNA synthesis kit (Promega), and subjected to PCR using SYBR Premix Ex Taq II (TaKaRa Bio Inc.). The primers used to amplify the MV genes were described previously (36). β-Actin mRNA was also quantified and used as an internal control (Fig. 2B). The levels of the viral transcripts (N, P, M, F, H, and L mRNAs, which show a transcriptional gradient [6, 7, 25]) were reduced ~10-fold in IC/Ed-P-Luci- and IC/Ed-L-Luci-infected cells, compared with the levels in IC323-Luci-infected cells (Fig. 2B). The viral transcript levels were even further reduced in IC/Ed-PL-Luci-infected cells, being about 100-fold lower than those in IC323-Luci-infected cells (Fig. 2B). These data show that the small-plaque-forming phenotype of the Edmonston strain is at least partly caused by reduced viral gene expression levels in cells. We also analyzed the activities of the P and L proteins using a minigenome assay (18, 19). All combinations of the P-
protein expression plasmid was omitted. Data are means ± standard deviations for triplicate samples. RPMI 1640 medium supplemented with 7.5% fetal bovine serum was used in transfections for triplicate samples.

At 6 h p.i., the culture media were replaced with RPMI 1640 medium supplemented with 7.5% fetal bovine serum. At 18 h p.i., mRNAs were purified from the cells, and the levels of N, P, M, F, H, and L mRNAs were determined by reverse transcription-quantitative PCR. Data are means ± standard deviations for triplicate samples. RLU, relative light units.

FIG. 2. Attenuated gene expression by recombinant MVs with the P and/or L genes of the Edmonston strain. (A) Confluent monolayers of various cell lines (Vero/hSLAM, CV1/hSLAM, HeLa/hSLAM, CHO/hSLAM, and B95a) and suspensions of nonadherent MT2 cells cultured in 24-well cluster plates were infected with 2.5 × 10⁴ PFU of IC323-Luci (circles), IC/Ed-P-Luci (diamonds), IC/Ed-L-Luci (triangles), and IC/Ed-PL-Luci (squares). After various intervals, the Renilla luciferase activities were measured. Data are means ± standard deviations for triplicate samples. RLU, relative light units. (B) Confluent monolayers of Vero/hSLAM cells in 6-cm culture plates were infected with 1.0 × 10⁴ PFU of IC323-Luci (light gray bars), IC/Ed-P-Luci (black bars), IC/Ed-L-Luci (dark gray bars), and IC/Ed-PL-Luci (white bars) and cultured in the presence of a fusion-blocking peptide. At 18 h p.i., mRNAs were purified from the cells, and the levels of N, P, M, F, H, and L mRNAs were determined by reverse transcription-quantitative PCR. Data are means ± standard deviations for triplicate samples. (C) Migenome assays. The method was described in detail elsewhere (19). Monolayers of CHO/hSLAM cells cultured in Opti-MEM on 24-well plates were infected with vTF7-3 at a multiplicity of infection of 0.5 and then transfected with 0.2 µg of p18MGFLuc01-wt-Le, 0.2 µg of pCAG-T7-IC-N, 0.3 µg of a P-protein expression plasmid (pCAG-T7-IC-PΔC or -Ed-PΔC), and 0.2 µg of an L-protein expression plasmid (pGEMCR-IC-L or -Ed-L) using Lipofectamine 2000 (Invitrogen). At 6 h p.i., the culture media were replaced with RPMI 1640 medium supplemented with 7.5% fetal bovine serum. At 48 h p.i., the firefly luciferase activities were measured. (-), L-protein expression plasmid was omitted. Data are means ± standard deviations for triplicate samples.

It is now clear that the P and L genes of the Ed-tag strain attenuate MV gene expression when introduced into recombinant virus genomes. To exclude possible growth-inhibitory effects caused by the inability of the Ed-tag V protein to block IFN signaling due to the Y110H and C272R substitutions (5, 8, 11, 22), the histidine and arginine residues at these positions were replaced with the corresponding residues of wild-type MV (tyrosine and cysteine, respectively), generating Ed-tag-VH110Y/R272C. An additional transcriptional unit for the P and L genes of the Ed-tag strain, especially when combined, reduced the plaque sizes of recombinant MVs (Fig. 3B).

Compared with the genomes of wild-type MV strains, the genomes of the Edmonston vaccine strains have three nucleotide substitutions (uracil-to-adenine, uracil-to-guanine, and uracil-to-cytosine changes at nucleotide positions 26, 42, and 50, respectively) in the genomic promoter (leader [Le]) region (16, 37). Using minigenome assays, these substitutions have been shown to enhance the promoter activity (16). To analyze the effects of these substitutions on virus infection, they were introduced into the IC323-Luci genome, generating IC/Ed-Le-Luci. The substitutions were also introduced into the IC/Ed-P-Luci, IC/Ed-Le-P-Luci, and IC/Ed-PL-Luci genomes, thereby generating IC/Ed-Le-P-Luci, IC/Ed-Le-Luci, and IC/Ed-Le-PL-Luci, respectively. The Renilla luciferase activities induced by IC323-Luci and IC/Ed-Le-Luci were compared in the 12 cell lines described above. No significant differences between the Renilla luciferase activities of IC323-Luci- and IC/Ed-Le-Luci-infected cells were observed for any of the 12 cell lines (Fig. 3A and data not shown). These data reveal that the three substitutions have neutral effects on the promoter function when introduced into the wild-type MV genome. Regardless of the presence or absence of these substitutions in the Le region, the P and L genes of the Ed-tag strain, especially when combined, reduced the plaque sizes of recombinant MVs (Fig. 3B).
Aliquots (1.0 ml) containing 1.5 \times 10^5 PFU of recombinant MVs (IC323-Luci, IC/Ed-L-Luci, and Ed-tag-V_{H110Y/R272C}-Luci) were injected into the peritoneal cavities of SLAM-knock-in mice crossed with mice lacking type I IFN receptor subunit 1 (IFNAR1-/-) SLAM-knock-in mice) (21). Eight mice were used for each recombinant MV. At 5 days p.i., the spleens were removed, homogenized using a BioMasher (Hi-Tech Inc.), and analyzed for their Renilla luciferase activities. The median Renilla luciferase activity in spleens from mice infected with Ed-tag-V_{H110Y/R272C}-Luci was ~10,000-fold lower than that in spleens from mice infected with wild-type IC323-Luci ($P < 0.001$; Fig. 4C). These data clearly indicate that the Edmonston strain is severely attenuated in IFNAR1-/- SLAM-knock-in mice. The median Renilla luciferase activity in spleens from IC/Ed-L-Luci-infected mice was ~200-fold lower than that in spleens from mice infected with wild-type IC323-Luci ($P < 0.001$; Fig. 4C). These data indicate that the L gene of the Edmonston strain contributes to the in vivo attenuation of the Edmonston strain.

Many viruses can be adapted to various cultured cells by passing in the cells, and it is empirically known that these adaptations often reduce virus virulence in natural host animals (15). Previous studies indicated that MV can adapt to grow in some cultured cells by acquiring specific substitutions in the receptor-binding hemagglutinin (H) and/or M proteins (43). Although some mechanisms by which these changes in the H and M proteins may cause attenuation have been proposed (30, 33), their contributions in vivo remain to be determined in animal models. Consistent with the present data, our previous study using a wild-type MV strain and its Vero cell-adapted strain suggested that substitutions introduced into the polymerase protein genes (L and P) during passages in Vero cells caused MV attenuation by reducing the transcriptional activities of viral polymerase (35). However, we observed at-
tenuated gene expression levels by the Edmonston strain only in virus infection analyses, not in minigenome assays. Bankamp et al. (2) also reported that the polymerase proteins of MV vaccine strains show higher transcriptional activities than those of wild-type MV strains when analyzed by minigenome assays. The detailed mechanisms of the attenuated gene expression induced by the polymerase protein genes of the Edmonston strain remain to be elucidated.

In conclusion, the present study demonstrates that the polymerase protein genes of the Edmonston strain contribute to its attenuated phenotype. Our data further show that assays using infectious recombinant viruses are crucial for understanding the contribution of each viral gene to virus replication and virulence and that the SLAM-knock-in mouse is a useful animal model for elucidating the attenuation mechanisms of MV vaccines.

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