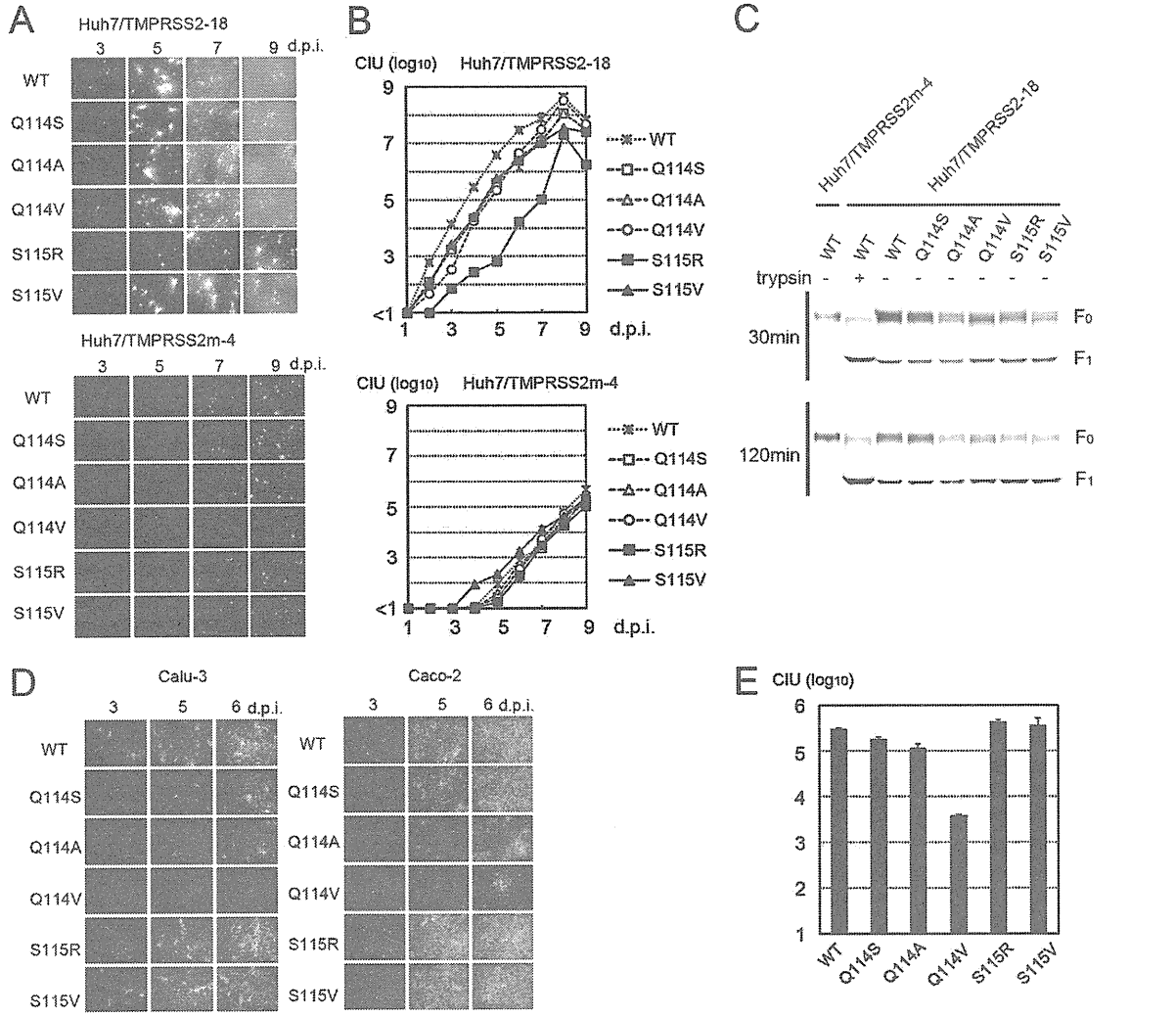

FIG 2 Analyses of recombinant SeV-RFP strains with mutations at P2 and P3 of the F protein. (A) Detection of wt- and mutant-SeV-RFP-infected cells. Monolayers of Huh7/TMPRSS2-18 and Huh7/TMPRSS2m-4 cells were infected with wt and mutant SeV-RFPs at an MOI of 0.01, cultured in the absence of trypsin, and observed daily using a fluorescence microscope. Data at 3, 5, 7, and 9 days postinfection are shown. (B) Replication kinetics of wt and mutant SeV-RFPs in Huh7/TMPRSS2-18 and Huh7/TMPRSS2m-4 cells. Cells were infected with wt and mutant SeV-RFPs at an MOI of 0.01, cultured in the absence of trypsin, and examined for their virus titers (CIU) daily. (C) Pulse-chase labeling, immunoprecipitation, and SDS-PAGE for detection of SeV F proteins. Huh7/TMPRSS2-18 and Huh7/TMPRSS2m-4 cells infected with wt or mutant SeV-RFP were pulse-labeled with [^{35}S]methionine for 15 min, cultured (chased) in normal medium for 30 or 120 min, and subjected to immunoprecipitation and SDS-PAGE for detection of the F proteins. (D) Detection of wt- and mutant-SeV-RFP-infected cells. Monolayers of Calu-3 and Caco-2 cells were infected with wt and mutant SeV-RFPs at an MOI of 0.001, cultured in the absence of trypsin, and observed daily using a fluorescence microscope. Data at 3, 5, and 6 days postinfection are shown. (E) Virus production in Calu-3 cells. Calu-3 cells were infected with wt and mutant SeV-RFPs at an MOI of 0.001 and examined for their virus titers (CIU) at 5 days postinfection.

responsible for activation of the IAV HA (22). Calu-3 and Caco-2 cells were infected with SeV-RFP(wt) and mutant SeVs. Both cell lines supported SeV-RFP(wt) multiplication well (Fig. 2D), similar to what occurred with IAV (20–22). All of the mutant SeVs also underwent multiple rounds of infection in both cell lines. However, unlike with Huh7/TMPRSS2-18 cells, the propagation of SeV-RFP/Q114V was severely deteriorated in these cell lines, whereas SeV-RFP/S115R spread well in these cell lines (Fig. 2D and E). Although not as dramatically, the other two mutants with mutations at P3 (SeV-RFP/Q114S and -RFP/Q114A) also spread less efficiently than SeV-RFP(wt) in these cell lines (Fig. 2D and E). Thus, the effects of each mutation observed in Calu-3 and Caco-2 cells were different from those observed in TMPRSS2-expressing Huh7 cells. Since Calu-3 and Caco-2 cells have more than one protease contributing to IAV HA activation, the SeV F protein may be activated mainly by proteases other than TMPRSS2 in these cells. Nevertheless, the glutamine residue at P3 was shown to be important for SeV multiplication in bronchial and intestinal epithelial cells.

The present study has demonstrated the importance of the P2 serine and P3 glutamine residues for SeV replication, but the roles of these residues remain unclear. Although our data showed little, if any, effects of the mutations on F protein cleavage by TMPRSS2, these residues may modulate the protease's specificity or sensitiv-

ity for the F protein. Many proteases have been shown to activate respiratory viruses. Trypsin is commonly used for the propagation of IAV, HMPV, SeV, HPIVs, and coronaviruses in cultured cells. Mini-plasmin found in bronchial epithelia (23), tryptase Clara from rat lungs (24, 25), mast cell tryptase from porcine lungs (26), and factor Xa from chicken allantoic fluid (26) were shown to proteolytically activate IAV and SeV. Plasmin also activates IAV through unique mechanisms by which plasminogen is captured by the neuraminidase surface glycoprotein or host cell annexin II is incorporated into virions (27–29). Recent studies have added a new class of proteases, transmembrane serine proteases (TTSPs), to the list of IAV- or SeV-activating proteases (2). TMPRSS2, HAT (TMPRSS11D), and TMPRSS4 were shown to activate seasonal IAV strains (3, 4, 21, 22). However, it remains totally unknown which proteases contribute mainly to respiratory virus pathogenesis. Although multiple proteases may contribute to the virus' spread *in vivo*, membrane-anchored proteases have an advantage for processing target substrates at a specific location with a minimum concentration (30, 31). Indeed, our previous study demonstrated that a marginal level of TMPRSS2 expression was sufficient for intracellular cleavage of the IAV HA and HMPV F proteins (7). To date, the biological and physiological functions of TMPRSS2 have been poorly elucidated. Although a previous study suggested that TMPRSS2 regulates the Na^+ current (32), the physiological importance of this is uncertain, because TMPRSS2 knockout mice showed no developmental, physiological, or pathological changes from wild-type mice (33). A final conclusion awaits *in vivo* analyses for the contribution of TMPRSS2 to viral pathogenesis. However, the accumulated evidence has revealed that TMPRSS2 acts as an activating protease for a broad range of respiratory viruses.

ACKNOWLEDGMENTS

We thank N. Ito and M. Sugiyama for providing the BHK/T7-9 cells. We also thank all the members of Department of Virology 3, NIID, Japan, for technical support.

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare of Japan and by a grant from The Takeda Science Foundation.

REFERENCES

1. Kido H, Okumura Y, Takahashi E, Pan HY, Wang S, Chida J, Le TQ, Yano M. 2008. Host envelope glycoprotein processing proteases are indispensable for entry into human cells by seasonal and highly pathogenic avian influenza viruses. J. Mol. Genet. Med. 3:167–175.
2. Choi SY, Bertram S, Glowacka I, Park YW, Pohlmann S. 2009. Type II transmembrane serine proteases in cancer and viral infections. Trends Mol. Med. 15:303–312.
3. Chaipan C, Kobasa D, Bertram S, Glowacka I, Steffen I, Tsegaye TS, Takeda M, Bugge TH, Kim S, Park Y, Marzi A, Pohlmann S. 2009. Proteolytic activation of the 1918 influenza virus hemagglutinin. J. Virol. 83:3200–3211.
4. Bottcher E, Matrosovich T, Beyerle M, Klenk HD, Garten W, Matrosovich M. 2006. Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium. J. Virol. 80: 9896–9898.
5. Hamilton BS, Gludish DW, Whittaker GR. 2012. Cleavage activation of the human-adapted influenza virus subtypes by matriptase reveals both subtype and strain specificities. J. Virol. 86:10579–10586.
6. Beaulieu A, Gravel E, Cloutier A, Marois I, Colombo E, Desilets A, Verreault C, Leduc R, Marsault E, Richter MV. 2013. Matriptase proteolytically activates influenza virus and promotes multicycle replication in the human airway epithelium. J. Virol. 87:4237–4251.
7. Shirogane Y, Takeda M, Iwasaki M, Ishiguro N, Takeuchi H, Nakatsu Y, Tahara M, Kikuta H, Yanagi Y. 2008. Efficient multiplication of human metapneumovirus in Vero cells expressing the transmembrane serine protease TMPRSS2. J. Virol. 82:8942–8946.
8. Bertram S, Glowacka I, Muller MA, Lavender H, Gnirss K, Nehlmeier I, Niemeyer D, He Y, Simmons G, Drosten C, Soilleux EJ, Jahn O, Steffen I, Pohlmann S. 2011. Cleavage and activation of the severe acute respiratory syndrome coronavirus spike protein by human airway trypsin-like protease. J. Virol. 85:13363–13372.
9. Glowacka I, Bertram S, Muller MA, Allen P, Soilleux E, Pfefferle S, Steffen I, Tsegaye TS, He Y, Gnirss K, Niemeyer D, Schneider H, Drosten C, Pohlmann S. 2011. Evidence that TMPRSS2 activates the severe acute respiratory syndrome coronavirus spike protein for membrane fusion and reduces viral control by the humoral immune response. J. Virol. 85:4122–4134.
10. Shulla A, Heald-Sargent T, Subramanya G, Zhao J, Perlman S, Gallagher T. 2011. A transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus receptor and activates virus entry. J. Virol. 85:873–882.
11. Matsuyama S, Nagata N, Shirato K, Kawase M, Takeda M, Taguchi F. 2010. Efficient activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane protease TMPRSS2. J. Virol. 84:12658–12664.
12. Kawase M, Shirato K, van der Hoek L, Taguchi F, Matsuyama S. 2012. Simultaneous treatment of human bronchial epithelial cells with serine and cysteine protease inhibitors prevents severe acute respiratory syndrome coronavirus entry. J. Virol. 86:6537–6545.
13. Gierer S, Bertram S, Kaup F, Wrensch F, Heurich A, Kramer-Kuhl A, Welsch K, Winkler M, Meyer B, Drosten C, Dittmer U, von Hahn T, Simmons G, Hofmann H, Pohlmann S. 2013. The spike protein of the emerging betacoronavirus EMC uses a novel coronavirus receptor for entry, can be activated by TMPRSS2 and is targeted by neutralizing antibodies. J. Virol. 87:5502–5511.
14. Karron RA, Collins PL. 2007. Parainfluenza viruses, p 1497–1526. *In* Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (ed), Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
15. Kato A, Sakai Y, Shioda T, Kondo T, Nakanishi M, Nagai Y. 1996. Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative or positive sense. Genes Cells 1:569–579.
16. Hasan MK, Kato A, Shioda T, Sakai Y, Yu D, Nagai Y. 1997. Creation of an infectious recombinant Sendai virus expressing the firefly luciferase gene from the 3′ proximal first locus. J. Gen. Virol. 78(Part 11):2813–2820.
17. Bottcher-Friebertshauser E, Freuer C, Sielaff F, Schmidt S, Eickmann M, Uhlendorff J, Steinmetzer T, Klenk HD, Garten W. 2010. Cleavage of influenza virus hemagglutinin by airway proteases TMPRSS2 and HAT differs in subcellular localization and susceptibility to protease inhibitors. J. Virol. 84:5605–5614.
18. Gosalia DN, Salisbury CM, Ellman JA, Diamond SL. 2005. High throughput substrate specificity profiling of serine and cysteine proteases using solution-phase fluorogenic peptide microarrays. Mol. Cell. Proteomics 4:626–636.
19. Gosalia DN, Salisbury CM, Maly DJ, Ellman JA, Diamond SL. 2005. Profiling serine protease substrate specificity with solution phase fluorogenic peptide microarrays. Proteomics 5:1292–1298.
20. Zhirnov O, Klenk HD. 2003. Human influenza A viruses are proteolytically activated and do not induce apoptosis in CACO-2 cells. Virology 313:198–212.
21. Bottcher-Friebertshauser E, Stein DA, Klenk HD, Garten W. 2011. Inhibition of influenza virus infection in human airway cell cultures by an antisense peptide-conjugated morpholino oligomer targeting the hemagglutinin-activating protease TMPRSS2. J. Virol. 85:1554–1562.
22. Bertram S, Glowacka I, Blazejewska P, Soilleux E, Allen P, Danisch S, Steffen I, Choi SY, Park Y, Schneider H, Schughart K, Pohlmann S. 2010. TMPRSS2 and TMPRSS4 facilitate trypsin-independent spread of influenza virus in Caco-2 cells. J. Virol. 84:10016–10025.
23. Murakami M, Towatari T, Ohuchi M, Shiota M, Akao M, Okumura Y, Parry MA, Kido H. 2001. Mini-plasmin found in the epithelial cells of bronchioles triggers infection by broad-spectrum influenza A viruses and Sendai virus. Eur. J. Biochem. 268:2847–2855.
24. Kido H, Yokogoshi Y, Sakai K, Tashiro M, Kishino Y, Fukutomi A, Katunuma N. 1992. Isolation and characterization of a novel trypsin-like protease found in rat bronchiolar epithelial Clara cells. A possible activator of the viral fusion glycoprotein. J. Biol. Chem. 267:13573–13579.

25. **Tashiro M, Yokogoshi Y, Tobita K, Seto JT, Rott R, Kido H.** 1992. Tryptase Clara, an activating protease for Sendai virus in rat lungs, is involved in pneumopathogenicity. J. Virol. **66**:7211–7216.
26. **Chen Y, Shiota M, Ohuchi M, Towatari T, Tashiro J, Murakami M, Yano M, Yang B, Kido H.** 2000. Mast cell tryptase from pig lungs triggers infection by pneumotropic Sendai and influenza A viruses. Purification and characterization. Eur. J. Biochem. **267**:3189–3197.
27. **Goto H, Kawaoka Y.** 1998. A novel mechanism for the acquisition of virulence by a human influenza A virus. Proc. Natl. Acad. Sci. U. S. A. **95**:10224–10228.
28. **Lazarowitz SG, Goldberg AR, Choppin PW.** 1973. Proteolytic cleavage by plasmin of the HA polypeptide of influenza virus: host cell activation of serum plasminogen. Virology **56**:172–180.
29. **LeBouder F, Morello E, Rimmelzwaan GF, Bosse F, Pechoux C, Delmas B, Riteau B.** 2008. Annexin II incorporated into influenza virus particles supports virus replication by converting plasminogen into plasmin. J. Virol. **82**:6820–6828.
30. **Hooper JD, Clements JA, Quigley JP, Antalis TM.** 2001. Type II transmembrane serine proteases. Insights into an emerging class of cell surface proteolytic enzymes. J. Biol. Chem. **276**:857–860.
31. **Antalis TM, Buzza MS, Hodge KM, Hooper JD, Netzel-Arnett S.** 2010. The cutting edge: membrane-anchored serine protease activities in the pericellular microenvironment. Biochem. J. **428**:325–346.
32. **Donaldson SH, Hirsh A, Li DC, Holloway G, Chao J, Boucher RC, Gabriel SE.** 2002. Regulation of the epithelial sodium channel by serine proteases in human airways. J. Biol. Chem. **277**:8338–8345.
33. **Kim TS, Heinlein C, Hackman RC, Nelson PS.** 2006. Phenotypic analysis of mice lacking the Tmprss2-encoded protease. Mol. Cell. Biol. **26**:965–975.

Journal of Vaccines & Immunization

Nagata S et al., J Vaccines Immun 2013, 1(2): 13-21
http://dx.doi.org/10.14312/2053-1273.2013-3

Original research

Open Access

Comparison of the live attenuated mumps vaccine (Miyahara strain) with its preattenuated parental strain

Nagata S[1], Maedera T[1], Nagata N[2], Kidokoro M[1], Takeuchi K[3], Kuranaga M[4], Takedaa M[1] and Kato A[1*]

[1] Department of Virology III, National Institute of Infectious Diseases, Musashi-Murayama, 208-0011, Japan
[2] Department of Pathology, National Institute of Infectious Diseases, Musashi-Murayama, 208-0011, Japan
[3] Department of Infection Biology, Division of Biomedical Science, Faculty of Medicine, University of Tsukuba, Tsukuba, 305-0006, Japan
[4] The Chemo-Sero-Therapeutic Research Institute (KAKETSUKEN), Kumamoto, 860-0083, Japan

Abstract

Live attenuated mumps vaccines have been developed by passaging the field isolates in cells that differ from the natural host. To study mumps viral (MuV) attenuation at the genomic level, we compared a live attenuated mumps vaccine (Miyahara strain) to its preattenuated parental strain. These two strains exhibited several phenotypic differences. The parental strain grew faster, reached a higher titer, and formed larger plaques and syncytia in Vero cells compared to the vaccine strain. In addition, intracranial injection of parental strain in neonatal rats resulted in greater ventricular enlargement than that caused by the vaccine strain. Four nucleotide changes leading to amino acid substitutions were found between the two viral genomes. One change is present in the N and L genes, respectively, and two in the F gene. The fusogenic activity of the cloned parental F gene evaluated by a cell-cell fusion assay was weaker than that of the vaccine F gene, and did not correspond to the activity caused by the living parental and vaccine strains. The transcriptional activities of N and L proteins were monitored by a CAT minigenome assay. Cloned parental N gene produced almost the same CAT activity as vaccine N, and cloned parental L gene produced significantly higher CAT activity than vaccine L. Thus among four nucleotide changes, the three occurring in N and F were not found to relate to the viral outcome, but we confirmed that at least one change in the L protein was involved in the growth phenotype of parental MuV.

Keywords: attenuation; fusion; mumps virus; neurovirulence; replication; vaccine; Miyahara strain

Introduction

Mumps is an acute, contagious vaccine-preventable disease caused by the mumps virus (MuV). A mumps epidemic occurs every 3 to 5 years in unvaccinated populations, and humans are the only natural host for MuV infection. MuV is transmitted by the respiratory route and replicates primarily in the nasal epithelium, leading to viremia, and it triggers secondary viral replication [1]. Up to 30% of MuV infections are asymptomatic or show only nonspecific respiratory symptoms. In 70% to 90% of symptomatic infections, MuV invasion of glandular epithelium results in mumps parotitis. MuV enters the cerebrospinal fluid (CSF) during the viremia. CSF pleocytosis leads to a form of aseptic meningitis that occurs in about 10% of mumps cases. More serious complications, such as deafness and encephalitis, occur at a low frequency [1].

MuV belongs to the genus *Rubulavirus* of the family *Paramyxoviridae* and has a negative-strand nonsegmented RNA genome of 15,384 bases with seven genes encoding eight proteins: the nucleocapsid (N), V, phospho (P), matrix (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN), and large (L) proteins. The N, P, and L proteins participate in viral replication and, together with the genomic RNA, form the ribonucleocapsid (RNP). In the virion, RNP is surrounded by a host-derived membrane undercoated by the M protein. The HN and F

***Corresponding author:** Atsushi Kato, Division of Radiological Protection and Biology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo, 162-8640, Japan, Tel.: 81 3 5285 1111, ext. 2421; Fax: 81 3 5285 1194; E-mail: akato@niid.go.jp

Received 22 April 2013 *Revised* 15 July 2013 *Accepted* 22 July 2013 *Published* 29 July 2013

Citation: Nagata S, Maedera T, Nagata N, Kidokoro M, Takeuchi K, Kuranaga M, Takedaa M, Kato A (2013) Comparison of the live attenuated mumps vaccine (Miyahara Strain) with its preattenuated parental strain. J Vaccines Immun 1: 13-21. doi:10.14312/2053-1273.2013-3

proteins projected onto the virion surface are responsible for cell binding and the entry of MuV. The functions of V and SH are less well understood [2–5]. The SH gene sequence is variable and is therefore used as the basis of MuV genotyping [6–8].

Live attenuated MuV vaccines have been developed by adapting the virus for different species of host or for different conditions of temperature, after repeated trial and error. The first vaccine, Jeryl Lynn strain, was introduced into the USA in 1967 and contributed to a dramatic reduction in mumps infection rates. To date, more than 10 vaccines have been produced. Some vaccines were found to retain a limited pathogenicity [9–11], and some tend to lose immunogenicity because of over-attenuation [12]. Five vaccines were developed in Japan (Urabe [13], Hoshino [14], Torii, Miyahara and NK-M46), and they were found to cause aseptic meningitis, although much less frequently than the natural infection rate [15, 16]. The virulence of MuV has recently been studied using the reverse genetic techniques established for Jeryl Lynn strain cDNA [17]. The genetic basis for attenuation has been vigorously studied [18–23], but a common genetic factor for MuV attenuation has not yet been identified.

In this study, we compared a Japanese mumps vaccine, Miyahara strain, with its parental strain in the preattenuated position to investigate the molecular basis of attenuation. The parental strain apparently has a different phenotype. The full genome sequences of the parental strain and the vaccine revealed four nucleotide substitutions. Those changes accompany the amino acid substitution, and one amino acid change occurring in the L protein was linked to replication capacity *in vitro*.

Materials and methods

Viruses

The MuV Miyahara isolate was originally obtained from a mumps patient in 1970 in Japan. A passage history of the Miyahara strain is indicated in Figure 1. The Miyahara field isolate had been lost, but the strain that locates on the preadapted position of chicken embryo fibroblasts (CE) passages (exact position was not known) was retrieved from the freezer of the National Institute of Infectious Diseases, Japan and used in this study as a parental strain with the approval of the Kaketsuken (the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan). We purchased the live attenuated Miyahara MuV vaccine (lot 305) from the Chemo-Sero-Therapeutic Research Institute. Wild-type MuVodate-1 [24,25] and 02-49 were kindly provided by Dr. H. Saito (Akita Prefectural Institute of Public Health, Akita, Japan) and Dr. Y. Matsui (Niigata Prefectural Institute of Public Health and Environmental Science, Niigata, Japan), respectively. In all virus stocks used in this study, defective interfering particles that might influence the results were not found.

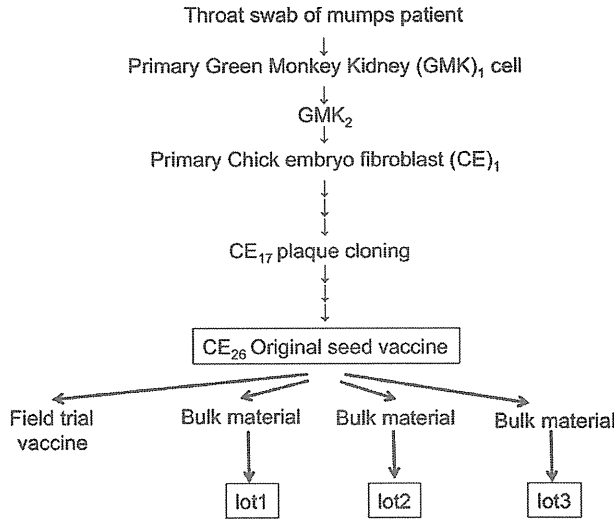

Figure 1 Passage history of the MuV Miyahara strain. MuV originally isolated from a throat swab of a 4-year old female mumps patient in primary green monkey kidney cells is shown as GMK_1. Another passage in the same GMK cells is shown as GMK_2. Passages in the primary cell culture of chick embryo fibroblasts are shown as CE. At the 17th passage of CE (CE_{17}), MuV was plaque purified. The test lot produced from CE_{26} was administered as the field trial vaccine. CE_{26} was licensed as the original seed for a live attenuated mumps vaccine, the Miyahara strain, in 1985 in Japan. The exact position of the Miyahara parental strain used in this study is not mapped due to the loss of records. Three bulk materials (lots 1, 2, and 3) have been produced, and the vaccine (lot 305) used in this study was produced from bulk lot 3.

Cell cultures and virus infection

Vero cells were grown in minimal essential medium (MEM) supplemented with 5% (v/v) bovine serum (BS) and inoculated with parental or vaccine strains at a multiplicity of infection (m.o.i.) of 0.01 cell infection unit (CIU)/cell unless otherwise noted. BHK-T7 cells [26] were kindly supplied by Dr. N. Ito (Gifu University, Gifu, Japan), and were maintained in Glasgow-MEM (Life Technologies Japan, Tokyo) containing 5% fetal BS.

For the growth kinetics study of parental and vaccine strains, 0.1 mL of culture supernatant was harvested daily until 5 days post-infection. The virus titer was then measured at least five times in each sample. MuV strains were titrated using Vero cells. Briefly, a diluted series of MuV was inoculated onto confluent cells in a 12-well plate after the removal of medium. After adsorption for 1 h, 1 mL of medium was added to each well. Thirty-six hours later, infected cells were treated with rabbit anti-MuV hyperimmune serum followed by fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin G. FITC-positive cells were measured, and the virus titer (CIU/ml) was calibrated.

For plaque formation, MuV was inoculated onto confluent Vero cells in a 6-well plate. After adsorption for 1 h, 1.0% (w/v) agarose in MEM containing 0.5% BS was overlaid on the cell culture and incubated at 37°C for 8 days. An agarose in MEM containing 0.01% (w/v) Neutral Red solution

(Sigma-Aldrich Japan, Tokyo) was again overlaid onto the first agarose layer and incubated for several hours until cells were stained. For the syncytium formation, MuV was inoculated onto cells at an input m.o.i. of 0.01 CIU/cell. Two days later, syncytia were observed by treating the cells with rabbit anti-MuV hyperimmune serum followed by FITC-conjugated anti-rabbit immunoglobulin G. FITC-positive fused cells were then counted.

Neurovirulence assay

Fertilized Lewis rats were purchased from Charles River Laboratories Japan (Yokohama, Japan). One-day-old littermates were inoculated intracranially with 20 μL of each MuV strain containing 100 CIU or phosphate-buffered saline (PBS) and raised with their respective dams. This experiment was performed according to the Institutional Animal Care and Use Committee regulations (Permission number: 211072). Under isoflurane anesthetic inhalation, these rats were sacrificed 30 days after the inoculation. Their heads were removed and fixed in 10% buffered formalin solution. Hydrocephalus, characterized by the excessive accumulation of CSF in the cerebral ventricles, was scored as described [27]. The sizes of the brain and lateral ventricles in a digital picture were measured using ImageJ software (National Institutes of Health, Bethesda, MD). The neurovirulence score for each strain is indicated by the mean score for all brains within an inoculated group. We analyzed differences in the scores using Student's t-test as described [19, 27].

PCR cloning and sequence analysis

The genome of MuV was extracted from the viral fluid using the High Pure Viral RNA Kit (Roche Diagnostics, Tokyo). Extracted RNAs were reverse-transcribed and amplified using the PrimeScript One Step RT-PCR Kit (Takara Bio, Otsu, Japan) with five sets of specific primer pairs that cover the entire sequence of the Miyahara vaccine strain as registered in the DNA database (accession number AB040874). These primer pairs amplified five regions that partially overlapped each other: 1(1-4592, the numericals in parentheses indicate the nucleotides from the 3' end), 2(3051-7413), 3(6049-10233), 4(8516-12935), and 5(11328-15384).

The amplified fragments were cloned into the pCR2.1 vector (Life Technologies Japan), yielding pCR-1, pCR-2, pCR-3, pCR-4, and pCR-5, respectively. We sequenced plasmids obtained from three independent colonies of transformed *E. coli*. Both termini of the genomes were amplified using the 5' and 3' RACE System (Life Technologies Japan). Nucleotide sequences were read using a BigDye Terminator v3.1 cycle sequencing kit with a 3130xl Genetic Analyzer (Life Technologies Japan). The final sequence was determined if the three sequences obtained from independent clones were identical. If these were not concordant, sequences were determined by the RT-PCR sequencing of bulk RNA. Obtained sequences were registered in the DNA database (accession number AB744048 and AB744049 for Miyahara vaccine lot 305 and parental strain, respectively).

The sequence of the Miyahara vaccine (AB744048) was not identical to the previously registered sequence (AB040874); it had a few nucleotide changes. We ignored these changes in our further studies as an error of the previous sequence, because these nucleotides were not found in the vaccine (AB744048) or in the parental strain (AB744049). The N, P, and L genes of each strain were cloned into the downstream site of the T7 promoter in a pGEM-3 vector (Promega, Tokyo), yielding pGEM-N, pGEM-P, and pGEM-L. The F and HN genes were cloned into the downstream site of the EF-BOS promoter in the pKS336 vector (AF403737, kindly provided by Dr. K. Sakai, National Institute of Infectious Diseases, NIID, Japan), yielding pKS-F and pKS-HN.

Fusion assay

One microgram each of pKS-F, pKS-HN, and pDsRed plasmids (Clontech, Takara Bio, Shiga, Japan) was cotransfected into confluent Vero cells using FuGENE 6 (Promega, Tokyo). Transfected cells were cultured for 24 h at 37°C or 39.5°C. Fused cells were observed by inverted fluorescent microscopy (AxioVert, Carl Zeiss Japan, Tokyo) and digitally captured using a CCD camera (AxioCam, Carl Zeiss Japan). The sizes of fused cells in the digital picture were scored using ImageJ software.

CAT minigenome assay

BHK-T7 cells, a baby hamster kidney (BHK) cell line stably expressing T7 polymerase [26], were seeded in 12-well plates at the semi-confluent level. Unless otherwise noted, 0.2 μg of pGEM-N, 0.2 μg of pGEM-P, 0.8 μg of pGEM-L, and 1.0 μg of pMuV-CAT were transfected using Lipofectamine 2000 (Life Technologies Japan). The plasmid pMuV-CAT is designed to produce a MuV-negative sense minigenome that codes the chloramphenicol acetyltransferase (CAT) reporter gene at an antisense manner [17]. CAT activity was assayed using the FAST CAT Chloramphenicol Acetyltransferase Assay Kit (Molecular Probes, Eugene, OR). Samples and the CAT reference standard were applied on a silica thin layer chromatography plate (Whatman, GE Healthcare Japan, Tokyo) and developed with chloroform:methanol (9:1) solvent.

Acetylation images were captured digitally using the luminescent image analyzer LAS-3000 (Fujifilm, GE Healthcare Japan). CAT activities were semiquantitatively indicated as a score by adding up the digital intensity of acetylated products at hydroxyl position 1 or 3 of chloramphenicol (CAP) and twice the intensity of those acetylated at both 1 and 3 hydroxyl positions, using ImageJ software. The experiments were performed in triplicate,

basically as described [17] except for our use of the non-radioisotope detection method.

Results

Characteristics of the Miyahara parental strain and vaccine in cells

Live attenuated MuV vaccines have been developed by adapting the virus for different species of host or for different conditions of temperature, after repeated trial and error. Five vaccines were developed in Japan through the adaptation to CE. During the vaccines' creation, candidates were designed to have the characteristic marker producing a smaller plaque than the parental field isolate in Vero cells. We compared the phenotypes of the preattenuated Miyahara parental strain to the live attenuated Miyahara commercial vaccine (Figure 1). The plaques caused by the parental strain were larger than those produced by the vaccine (Figure 2A). The parental plaque consisted of a clear edge and a colorless inside, but the vaccine plaque was unclear and turbid. This morphological character corresponded well with the developmental marker of the vaccine.

Differences in plaque sizes often reflect the viral capacity for fusogenicity, growth, or cytopathogenicity. We thus compared the syncytia of the two viruses at two days post-infection by staining the infected Vero cells with the anti-MuV antibody. The parent strain showed larger syncytia in Vero cells than the vaccine did (Figure 2B). Viral growth at the multistep condition (m.o.i. of 0.01) was then compared at three different temperatures (32°, 37°, and 39.5°C). The results from a representative of three independent experiments runs are shown in Figure 2C. At 32°C, the parental strain and the vaccine grew at almost the same growth rate.

In the higher temperature conditions, however, the parental strain increased faster than the vaccine and reached a higher titer compared to the vaccine. The virus titers of the parental strain at 3 days post-infection were 8.0×10^5 (32°C), 7.9×10^6 (37°C), and 3.0×10^7 (39.5°C) CIU/mL (Figure 2C, filled black arrows). These results indicated the increased growth phenotype of the parental strain with increasing temperature. In contrast, the vaccine grew almost identically in the three temperature

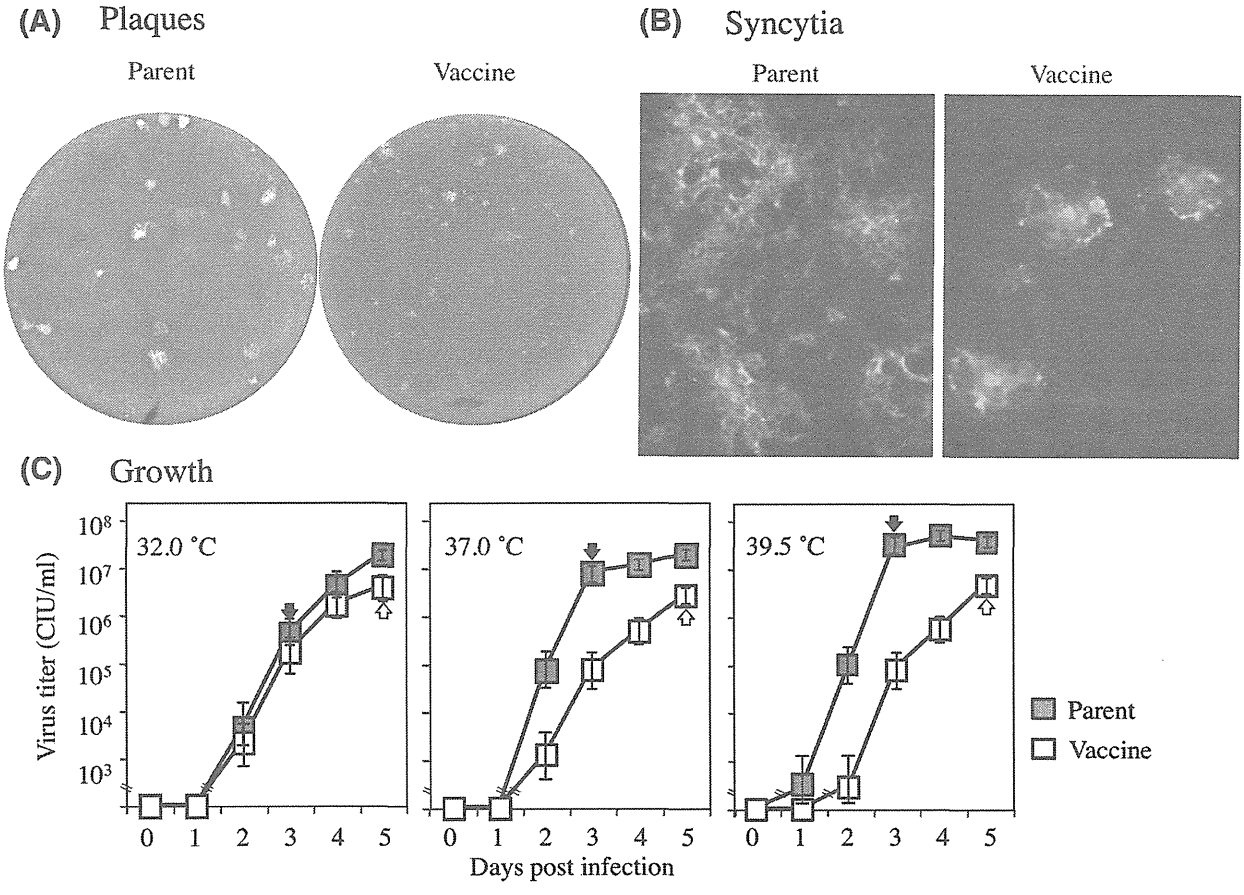

Figure 2 Phenotypic comparisons of parental strain and vaccine of MuV. Plaques produced on Vero cell culture (A). Syncytia found in Vero cell culture (B). Syncytia were reacted with rabbit anti-MuV hyperimmune serum as described in the materials and methods section. The growth of parental MuV and vaccine was compared under the temperature conditions indicated (C). A representative of three independent studies (32° and 37°C, 37° and 39.5°C, and 32° and 39.5°C) is shown. Closed boxes show the titer of parent MuV; open boxes show that of vaccine MuV. Each point indicates the mean CIU/mL titer of six calculations. Error bars: SEM.

conditions and reached identical titers (3.8×10^6 at 32°C, 2.7×10^6 at 37°C, and 4.3×10^6 CIU/mL at 39.5°C) at 5 days post-infection (Figure 2C, outlined white arrows). We tried to compare the viral growth at 42°C but failed to obtain growing viruses from the culture supernatant because of the high-temperature sensitivity of the Vero cells (data not shown).

Characteristics of neurovirulence in rats

Since humans are the only natural host for MuV, no routine method for assaying the pathogenicity of MuV has been established, although attempts have been made with the Cynomolgus monkey [28, 29], marmoset [30], and hamster [31]. We adopted a neurovirulence assay model using neonatal rats [27, 32]. In the present study, we injected two field isolates (Odate-I and 02-49) and PBS intracranially into littermates of rats as the positive and the negative controls along with the parental strain and vaccine (Figure 3). Odate-I was isolated from a meningitis patient during a mumps outbreak in 1993 with a high incidence of aseptic meningitis [24, 25], while 02-49 was isolated from a parotitis patient in 2002 in Japan.

The neurovirulence score for Odate-I was 17.54±4.12 (n=18) and for 02-49 was 7.44±3.22 (n=18). The decrease of neurovirulence observed in 02-49 was statistically significant (P<0.01). PBS-inoculated rats

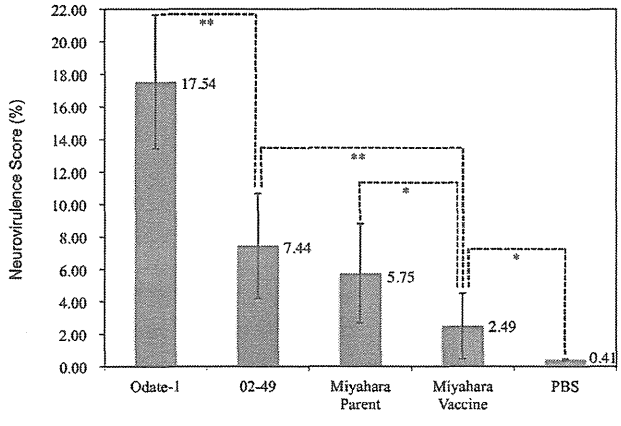

Figure 3 The severity of hydrocephalus in rats inoculated with the parental strain and vaccine of MuV. We tested the neurovirulence of parental MuV and vaccine as described in the materials and methods section. All bars and numerals represent the mean values of neurovirulence scores. Error bars: SEM. **, P<0.01; *, P<0.05). The Miyahara vaccine's score was 2.64±2.41(n=20), significantly lower than that of 02-49 (P=0.006<0.01) but higher than that of PBS (P=0.026<0.05).

did not show any apparent changes and the score was 0.41±0.05 (n=24). The Miyahara vaccine's score was 2.49±2.41 (n=20), significantly lower than that of 02-49 (P<0.01), indicating the avirulent phenotype of approved vaccine. The neurovirulence score of the parental strain was 5.75±3.05 (n=18). This score was higher than that of the vaccine (P=0.048<0.05), but no significant difference between the parental strain score and that of 02-49 was revealed (P=0.436). This result is in line with the finding that the Miyahara parental strain was moving toward adapting to CE cells and thus retaining the neurovirulence.

Differences in genome sequences

At this point, we had found some biologically significant differences between the Miyahara parent strain and the vaccine. A comparison of the genome sequence between the two strains was then necessary to better understand their biological functions at the molecular level. We cloned three independent fragments amplified from each genome and sequenced them carefully. Both termini were confirmed using the 3' and 5' RACE methods as described in the materials and methods section. Our analysis of the sequence data (AB744048 and AB744049) yielded four nucleotides that differed between the two strains: 1337 in the N gene, 5024 and 5129 in the F gene, and 14355 in the L gene (the numerals indicate the position from the 3' end). There was no deletion or insertion in the genome, and no nucleotide exchange had occurred in the noncoding region. All four exchanges were accompanied by the amino acid changes $1337N^{398}$(Val → Ile), $5024F^{160}$(Gln → Arg), $5129F^{195}$(Phe → Ser), and $14355L^{1973}$(Phe → Ser).

Fusogenicity of cells transfected with cloned F and HN genes

The F protein of MuV is responsible for the virus-cell and the cell-cell membrane fusion with the cooperation of the HN protein. To determine whether the amino acid exchanges in the F protein facilitated the fusion activity, we cotransfected plasmids coding each F and HN protein into Vero cells together with the pDsRed, and incubated them at 37°C or 39.5°C. The syncytia thus formed emitted red fluorescence (Figure 4). In marked contrast to the syncytia with live MuV-infected cells (Figure 2), the sizes of the syncytia associated with the parental F gene were smaller than those associated with the vaccine F gene.

The diameters of 50 randomly taken syncytia were measured and the histogram was drawn at 37°C and 39.5°C. The syncytia produced at 39.5°C were smaller than those obtained at 37°C. The temperature-independent syncytia formation by cloned F genes was the opposite of the higher growth rate of living parent MuV at 39.5°C compared to 37°C. These results showed that the vaccine phenotype was not explained by $5024F^{160}$(Gln → Arg) and $5129F^{195}$(Phe → Ser) mutations.

Minigenome activity of cloned genes

The P and L proteins form the viral RNA polymerase and the N protein surrounds the genomic RNA to form an

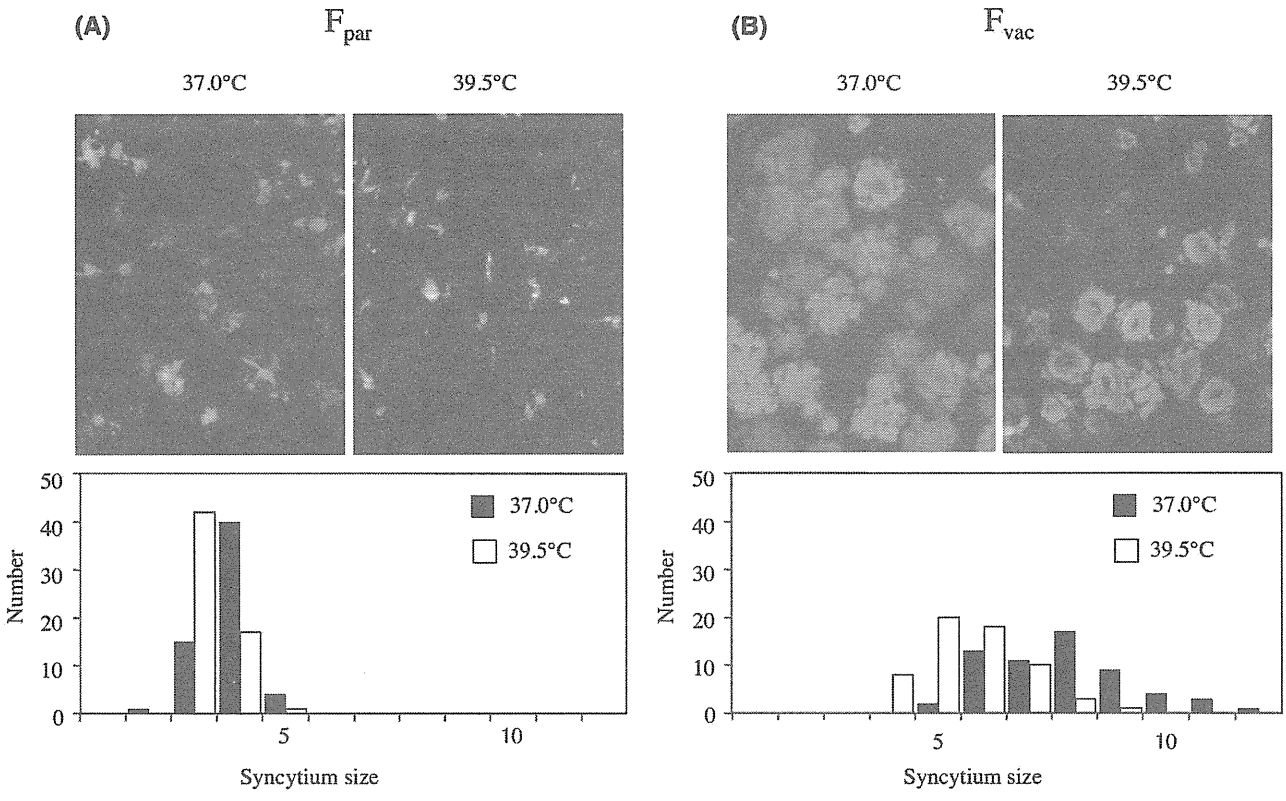

Figure 4 (A,B) Syncytium formation assay by cloned F and HN genes. Vero cell cultures were transfected with plasmids encoding cloned F gene from parent (F_{par}) or vaccine (F_{vac}) and HN gene together with pDsRed. Fused cells emitting fluorescence are shown. At 37°C (closed bar) or 39.5°C (open bar), the images of syncytia were digitally taken and the diameters were plotted as histograms.

active template. To evaluate the relevance of 1337N[398] (Val → Ile) and 14355L[1973](Phe → Ser) *in vitro*, we used a minigenome in which all MuV genes of the Miyahara strains were replaced with a CAT gene. CAT activity reflected the amount of CAT mRNA produced from the minigenome. We found no CAT activity in the experimental conditions when one of three proteins was depleted, but found that the use of 0.2 μg, 0.2 μg, and 0.4 μg of N, P, and L plasmids, respectively, resulted in the optimum CAT activity. Inputting the vaccine N (N_{vac}) or parental N (N_{par}) plasmid from 0 to 0.1 and 0.2 μg, the three clearer forms of acetylated products (1-, 3-, and 1,3-chloramphenicol (CAP)) were observed (Figure 5A). However, further input of the N plasmid (0.4 and 0.6 μg) did not result in an increase in acetylated products but rather their decrease. Maximum CAT activities were shown at 0.2 μg of N plasmid, and were almost the same between the two N plasmids. By the digital intensity of acetylated products, the amount of products was indicated as 34.1 (vaccine) and 35.1 (parent). These results showed that N_{vac} and N_{par} had no functional difference and that both were equivalent in the CAT assay. Any relevance of 1337N[398](Val → Ile) was not identified under this condition.

We then added various amounts of vaccine L (L_{vac}) or parental L (L_{par}) plasmid together with N and P plasmids. Too many or too few L plasmids did not result in high CAT activity (Figure 5B). The maximum CAT activity was found under the conditions in which 0.4 μg or 0.8 μg of L_{par} or L_{vac} was added. The digital intensity of acetylated products was indicated as 51.8 (parent) and 32.8 (vaccine). Higher CAT activity was observed in L_{par}-transfected cells than in L_{vac}-transfected cells. These results indicate that L_{par} and L_{vac} did not have the same effect and that L_{par} had 1.6-fold (51.8/32.8) higher transcription and replication capacities. We tested and confirmed this higher capacity of L_{par} to L_{vac} by measuring acetylated products in three independent experiments. Thus, 14355L[1973](Phe → Ser) was relevant under this condition.

Although an effect of the N mutation was not observed in the above experiment, it is possible that the mutation in the N gene might have an effect in combination with the mutation in the L gene. We therefore added various amounts of L_{vac} with a combination of fixed amounts of N_{vac}, and we also added L_{par} with a combination of fixed amounts of N_{par}. Higher CAT activity was observed in N_{par}/L_{par}-transfected cells than in N_{vac}/L_{vac}-transfected cells. Maximum CAT activities were obtained when 0.4 μg or 0.8 μg of L_{par} or L_{vac} was added. The scores shown by the digital intensity of acetylated products were 57.4 (parent) and 35.4 (vaccine) (data not shown). These results again

showed the importance of 14355L[1973](Phe → Ser), but in a comparison of N_{vac}/L_{vac} and N_{par}/L_{par} (57.4/35.4 = 1.6 fold) to N_{vac}/L_{vac} and N_{vac}/L_{par} (51.8/32.8 = 1.6 fold, Figure 5B), the fold ratio of vaccine to parent gave the same value. Similar results were also obtained when various amounts of N_{vac} were used in combination with fixed amounts of L_{vac} and when N_{par} was used in combination with fixed amounts of Lpar (data not shown). These findings show no contribution of 1337N[398] (Val → Ile) mutation on CAT activity in combination with L protein.

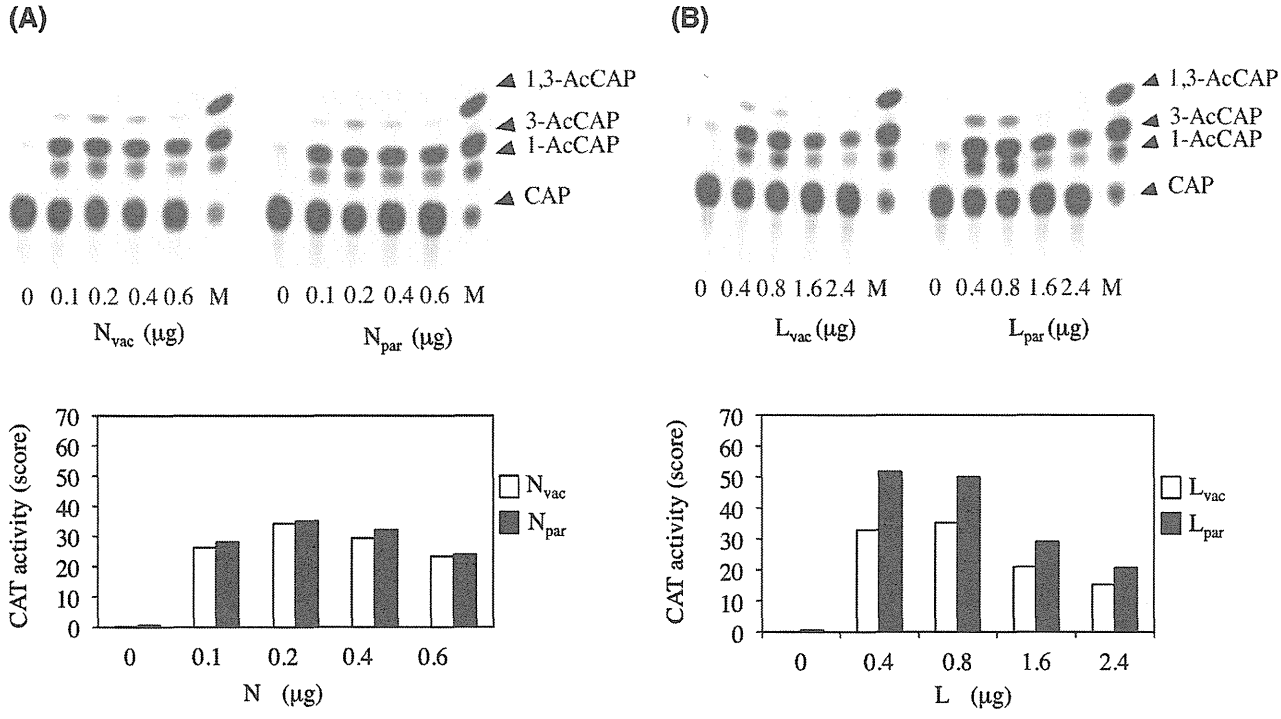

Figure 5 CAT minigenome assay for evaluating mutations in the N and L genes. Various amounts of the N gene from parent (N_{par}) or vaccine (N_{vac}) were transfected into the cells together with P and vaccine L (L_{vac}) genes and the CAT minigenome, as described in the materials and methods section (A). Various amounts of the L gene from parent (L_{par}) or vaccine (L_{vac}) were transfected together with P and N_{vac} genes and the CAT minigenome (B). The acetylated products developed in TLC plates are shown in the upper panel. The intensities of acetylated spots obtained from the digital photograph are indicated as the CAT activity bar graphs in the bottom panel. Open and closed bars show the results obtained by vaccine- and parent-derived gene transfection, respectively. Data are from a representative experiment performed in triplicate.

Discussion

Wild-type MuV exhibits neurovirulence, and even attenuated vaccines carry some risk of neurovirulence approved attenuated vaccines carry some risk of neurovirulence and can cause aseptic meningitis, although the frequency of its occurrence with the mumps vaccine is low compared to the natural infection. Approximate frequencies are specifically determined by data from administered vaccine strains [1, 9], which indicate some genetic factor dictating MuV's neurovirulence. The identification of such a gene or genes is thus important for developing a safe vaccine.

It was reported that the Urabe mumps vaccine is a mixture of viruses differing at amino acid 335 of the HN gene, and one form was associated with neurovirulence [33]. Some virological studies supported this finding [21, 22]. However, the complete nucleotide sequences of several stocks of the Urabe strain did not support this finding [34, 35], and reverse genetic work finally showed that HN[335] was not a crucial determinant of Urabe neurovirulence [36].

The Jeryl Lynn vaccine strain adapted to human neuronal cells had three amino acid substitutions in N[468](Phe → Pro), M[85](Val → Ala) and L[1165](Glu → Asp), and the wild-type 88-1961 strain adapted to CE fibroblasts had three amino acid substitutions in F[91](Ala/Thr → Thr), HN[466](Ser → Asn) and L[736](Ile → Val) [37]. Swapping of the respective gene from the Jeryl Lynn strain to the 88-1961 strain resulted in a gradual attenuation with the increase in the number of genes and yielded no critical gene for viral virulence, and swapping those from the wild-type to the Jeryl Lynn strain resulted in a gradual virulence to the contrary. [23]. These results suggest that there is no specific gene determining viral attenuation and neurovirulence, and that viral attenuation and neurovirulence are determined by a sum or a certain combination of noncritical genes in the context of viral life cycle.

We started to study MuV attenuation using one of

the Japanese vaccines, the Miyahara strain, and its preattanuated parental strain. We found that the plaque size, viral growth, temperature sensitivity, fusogenicity and neurovirulence of the two strains were different (Figurs 2 and 3). The genome sequences of the parental strain and vaccine were carefully determined by two-step experiments. In the first step, three independent cDNA clones of both strands were sequenced and the sequence was determined only when the results were identical. In a few non-concordant cases, sequences were determined by the RT-PCR sequencing of bulk RNA as a second step. The results indicated that each virus source was highly homogeneous.

Considering that the Jeryl Lynn vaccine contains JL2 and JL5 component stains which differ by over 400 nucleotides [38], it was surprising for us that these phenotypes were probably caused by four nucleotide exchanges between the genomes. Among the four exchanges, $5024F^{160}$(Gln → Arg) and $5129F^{195}$(Phe → Ser) seemed not to be related to the Miyahara phenotype but were epiphenomena (Figure 4). Moreover, $5129F^{195}$(Phe) is found among known mumps strains independent of vaccines or field isolates. For example, the Odate-1 strain used for the rat neurovirulence test as a virulent strain in the present study has $5129F^{195}$(Ser), while the Urabe strain approved for the mumps vaccine in Japan has $5129F^{195}$(Phe). The relevance of $5129F^{195}$(Phe) to low fusogenic activity has been reported in other strains [39,40]. The discrepancy in fusogenic activity between the living virus and the cloned F gene was not resolved in this study.

We found that $14355L_{par}{}^{1973}$(Phe) resulted in higher CAT activity than $14355L_{vac}{}^{1973}$(Ser), but that $1337N_{par}{}^{398}$ (Val → Ile) mutation did not result in a significant difference (Figure 5). We concluded that an amino acid substitution occurring in $14355L_{par}{}^{1973}$(Ser) is associated with attenuation by reducing replication and transcription activity. Six conserved L domains (I-VI) are known between two different families of *Mononegavirales, rhabdoviridae* and *paramyxoviridae*, and are postulated to constitute the specific enzymatic activities [41]. However, the amino acid change observed herein in L^{1973} does not belong to any of the conserved regions. How the amino acid change in L^{1973} might alter transcriptional activity is unknown. Another possibility also remains that the mutations contribute to viral attenuation in a way that the fusogenic or the minigenome assay *in vitro* does not capture. The importance of amino acid mutations during the attenuation process will be revisited as a next step using the reverse genetics technique in the context of viral multiplication in cells and of viral neuropathogenecity for neonatal rats.

Acknowledgements

We thank Y. Ami and Y. Suzaki (NIID) for their helpful support with animal experiments, M. Tahara (NIID) for providing the T7-expressing cells, and T. Kubota (NIID) for the helpful advice.

Funding

This work was supported by a grant from the Ministry of Health, Labour and Welfare, Japan.

Conflict of interest

All the authors declare that they have no conflict of interest.

References

[1] Rubin SA, Carbone KM (2011) Mumps, In: Longo DL, Fauci AS, Kasper DL, Hauser SL, Jamenson JL, Loscalzo J, eds. Harrison's Principles of Internal Medicine. 18th ed. New York: The McGaw Hill Companies Inc., p1608–1610.

[2] Malik T, Shegogue CW, Werner K, Ngo L, Sauder C, et al. (2011) Discrimination of mumps virus small hydrophobic gene deletion effects from gene translation effects on virus virulence. J Virol 85:6082–6085.

[3] Takeuchi K, Tanabayashi K, Hishiyama M, Yamada A (1996) The mumps virus SH protein is a membrane protein and not essential for virus growth. Virology 225:156–162.

[4] Xu P, Li Z, Sun D, Lin Y, Wu J, et al. (2011) Rescue of wild-type mumps virus from a strain associated with recent outbreaks helps to define the role of the SH ORF in the pathogenesis of mumps virus. Virology 417:126–136.

[5] Xu P, Luthra P, Li Z, Fuentes S, D'Andrea JA, et al. (2012) The V protein of mumps virus plays a critical role in pathogenesis. J Virol 86:1768–1776.

[6] Jin L, Rima B, Brown D, Orvell C, Tecle T, et al. (2005) Proposal for genetic characterisation of wild-type mumps strains: preliminary standardisation of the nomenclature. Arch Virol 150:1903–1909.

[7] Kidokoro M, Tuul R, Komase K, Nymadawa P (2011) Characterization of mumps viruses circulating in Mongolia: Identification of a novel cluster of genotype H. J Clin Microbiol 49:1917–1925.

[8] Takeuchi K, Tanabayashi K, Hishiyama M, Yamada A, Sugiura A (1991) Variations of nucleotide sequences and transcription of the SH gene among mumps virus strains. Virology 181:364–366.

[9] Bonnet MC, Dutta A, Weinberger C, Plotkin SA (2006) Mumps vaccine virus strains and aseptic meningitis. Vaccine 24:7037–7045.

[10] Rafiefard F, Johansson B, Tecle T, Orvell C (2005) Characterization of mumps virus strains with varying neurovirulence. Scand J Infect Dis 37:330–337.

[11] Rubin SA, Afzal MA (2011) Neurovirulence safety testing of mumps vaccines--historical perspective and current status. Vaccine 29:2850–2855.

[12] Ong G, Goh KT, Ma S, Chew SK (2005) Comparative efficacy of Rubini, Jeryl-Lynn and Urabe mumps vaccine in an Asian population. J Infect. 51:294–298.

[13] Yamanishi K, Takahashi M, Kurimura T, Ueda S, Minekawa Y, et al. (1970) Studies on live mumps virus vaccine. 3. Evaluation of newly developed live mumps virus vaccine. Biken J 13:157–161.

[14] Sasaki K, Higashihara M, Inoue K, Igarashi Y, Makino S (1976) Studies on the development of a live attenuated mumps virus vaccine. I. Attenuation of the Hoshino 'wild' strain of mumps virus. Kitasato Arch Exp Med 49:43–52.

[15] Galazka AM, Robertson SE, Kraiger A (1999) Mumps and mumps vaccine: a global review. Bull World Health Organ. 77:3–14.

[16] Nagai T, Okafuji T, Miyazaki C, Ito Y, Kamada M, et al. (2007) A comparative study of the incidence of aseptic meningitis in symptomatic natural mumps patients and monovalent mumps vaccine recipients in Japan. Vaccine 25:2742-2747.

[17] Clarke DK, Sidhu MS, Johnson JE, Udem SA (2000) Rescue of mumps virus from cDNA. J Virol 74:4831-4838.

[18] Lemon K, Rima BK, McQuaid S, Allen IV, Duprex WP (2007) The F gene of rodent brain-adapted mumps virus is a major determinant of neurovirulence. J Virol 81:8293-8302.

[19] Malik T, Sauder C, Wolbert C, Zhang C, Carbone KM, et al. (2007) A single nucleotide change in the mumps virus F gene affects virus fusogenicity in vitro and virulence in vivo. J Neurovirol 13:513-521.

[20] Malik TH, Wolbert C, Nerret L, Sauder C, Rubin S (2009) Single amino acid changes in the mumps virus haemagglutinin-neuraminidase and polymerase proteins are associated with neuroattenuation. J Gen Virol 90:1741-1747.

[21] Reyes-Leyva J, Baños R, Borraz-Argüello M, Santos-López G, Rosas N, et al. (2007) Amino acid change 335 E to K affects the sialic-acid-binding and neuraminidase activities of Urabe AM9 mumps virus hemagglutinin-neuraminidase glycoprotein. Microbes Infect 9:234-240.

[22] Santos-López G, Cruz C, Pazos N, Vallejo V, Reyes-Leyva J, et al. (2006) Two clones obtained from Urabe AM9 mumps virus vaccine differ in their replicative efficiency in neuroblastoma cells. Microbes Infect 8:332-339.

[23] Sauder CJ, Zhang CX, Link MA, Duprex WP, Carbone KM, et al. (2009) Presence of lysine at aa 335 of the hemagglutinin-neuraminidase protein of mumps virus vaccine strain Urabe AM9 is not a requirement for neurovirulence. Vaccine 27:5822-5829.

[24] Saito H, Takahashi Y, Harata S, Tanaka K, Sano T, et al. (1996) Isolation and characterization of mumps virus strains in a mumps outbreak with a high incidence of aseptic meningitis. Microbiol Immunol 40:271-275.

[25] Saito H, Takahashi Y, Harata S, Tanaka K, Sato H, et al. (1998) Cloning and characterization of the genomic RNA sequence of the mumps virus strain associated with a high incidence of aseptic meningitis. Microbiol Immunol 42:133-137.

[26] Ito N, Takayama-Ito M, Yamada K, Hosokawa J, Sugiyama M, et al. (2003) Improved recovery of rabies virus from cloned cDNA using a vaccinia virus-free reverse genetics system. Microbiol Immunol 47:613-617.

[27] Rubin SA, Pletnikov M, Taffs R, Snoy PJ, Kobasa D, et al. (2000) Evaluation of a neonatal rat model for prediction of mumps virus neurovirulence in humans. J Virol 74:5382-5384.

[28] Afzal MA, Marsden SA, Hull RM, Pipkin PA, Bentley ML, et al. (1999) Evaluation of the neurovirulence test of mumps vaccines. Biologicals 27:43-49.

[29] Maximova O, Dragunsky E, Taffs R, Snoy R, Cogan J, et al. (1996) Monkey neurovirulence test for live mumps vaccine. Biologicals 24:233-234.

[30] Saika S, Kidokoro M, Aoki A, Ohkawa T (2004) Neurovirulence of mumps virus: intraspinal inoculation test in marmosets. Biologicals 32:147-152.

[31] Wolinsky JS, Stroop WG (1978) Virulence and persistence of three prototype strains of mumps virus in newborn hamsters. Arch Virol 57:355-359.

[32] Rubin SA, Pletnikov M, Carbone KM (1998) Comparison of the Neurovirulence of a vaccine and a wild-type mumps virus strain in the developing rat brain. J Virol 72:8037-8042.

[33] Brown EG, Dimock K, Wright KE (1996) The Urabe AM9 mumps vaccine is a mixture of viruses differing at amino acid 335 of the hemagglutinin-neuraminidase gene with one form associated with disease. J Infect Dis 174:619-622.

[34] Amexis G, Fineschi N, Chumakov K (2001) Correlation of genetic variability with safety of mumps vaccine Urabe AM9 strain. Virology 287:234-241.

[35] Wright KE, Dimock K, Brown EG (2000) Biological characteristics of genetic variants of Urabe AM9 mumps vaccine virus. Virus Res 67:49-57.

[36] Sauder CJ, Zhang CX, Ngo L, Werner K, Lemon K, et al. (2011) Gene-specific contributions to mumps virus neurovirulence and neuroattenuation. J Virol 85:7059-7069.

[37] Rubin SA, Amexis G, Pletnikov M, Li Z, Vanderzanden J, et al. (2003) Changes in mumps virus gene sequence associated with variability in neurovirulent phenotype. J Virol 77:11616-11624.

[38] Chambers P, Rima BK, Duprex WP (2009) Molecular differences between two Jeryl Lynn mumps virus vaccine component strains, JL5 and JL2. J Gen Virol 90:2973-2981.

[39] Tanabayashi K, Takeuchi K, Okazaki K, Hishiyama M, Yamada A (1993) Identification of an amino acid that defines the fusogenicity of mumps virus. J Virol 67:2928-2931.

[40] Tanabayashi K, Takeuchi K, Hishiyama M, Yamada A (1994) Effect on fusion induction of point mutations introduced into the F protein of mumps virus. Virology 204:851-853.

[41] Poch O, Blumberg, BM, Bougueleret L, Tordo N (1990) Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. J Gen Virol 71:1153-1162.

Vaccine 31S (2013) B163–B175

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Review

A global regulatory science agenda for vaccines

Lindsay Elmgren[a], Xuguang Li[a], Carolyn Wilson[b,1], Robert Ball[b,1], Junzhi Wang[c], Klaus Cichutek[d], Michael Pfleiderer[d], Atsushi Kato[e], Marco Cavaleri[f], James Southern[g], Teeranart Jivapaisarnpong[h], Philip Minor[i], Elwyn Griffiths[j], Yeowon Sohn[k], David Wood[l,m,*,2]

[a] *Health Canada, Ottawa, Canada*
[b] *Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA*
[c] *National Institute for Food and Drug Control, Beijing, China*
[d] *Paul Ehrlich Institut, Langen, Germany*
[e] *National Institute of Infectious Diseases, Tokyo, Japan*
[f] *European Medicines Agency, London, UK*
[g] *Cape Town, South Africa*
[h] *Institute of Biological Products, Department of Medical Sciences, Ministry of Public Health, Bangkok, Thailand*
[i] *National Institute of Biological Standards and Control, Potters Bar, UK*
[j] *London, UK*
[k] *Korea Food and Drug Administration, Seoul, Republic of Korea*
[l] *World Health Organization, Geneva, Switzerland*
[m] *Quality Safety and Standards Team, Department of Immunization, Vaccines and Biologicals, World Health Organization, Avenue Appia 20, 1211 Geneva 27, Switzerland*

ARTICLE INFO

Article history:
Received 8 August 2012
Received in revised form 22 October 2012
Accepted 31 October 2012

Keywords:
Vaccine regulation
Clinical trials
Post-marketing surveillance
Vaccine quality
Correlates of immunity
Vaccine standardization

ABSTRACT

The Decade of Vaccines Collaboration and development of the Global Vaccine Action Plan provides a catalyst and unique opportunity for regulators worldwide to develop and propose a global regulatory science agenda for vaccines. Regulatory oversight is critical to allow access to vaccines that are safe, effective, and of assured quality. Methods used by regulators need to constantly evolve so that scientific and technological advances are applied to address challenges such as new products and technologies, and also to provide an increased understanding of benefits and risks of existing products. Regulatory science builds on high-quality basic research, and encompasses at least two broad categories. First, there is laboratory-based regulatory science. Illustrative examples include development of correlates of immunity; or correlates of safety; or of improved product characterization and potency assays. Included in such science would be tools to standardize assays used for regulatory purposes. Second, there is science to develop regulatory processes. Illustrative examples include adaptive clinical trial designs; or tools to analyze the benefit-risk decision-making process of regulators; or novel pharmacovigilance methodologies. Included in such science would be initiatives to standardize regulatory processes (e.g., definitions of terms for adverse events [AEs] following immunization). The aim of a global regulatory science agenda is to transform current national efforts, mainly by well-resourced regulatory agencies, into a coordinated action plan to support global immunization goals. This article provides examples of how regulatory science has, in the past, contributed to improved access to vaccines, and identifies gaps that could be addressed through a global regulatory science agenda. The article also identifies challenges to implementing a regulatory science agenda and proposes strategies and actions to fill these gaps. A global regulatory science agenda will enable regulators, academics, and other stakeholders to converge around transformative actions for innovation in the regulatory process to support global immunization goals.

* Corresponding author. Tel.: +41 22 791 4050.
E-mail address: woodd@who.int (D. Wood).

[1] Additional CBER scientists who contributed to the document were: Dale Horne, Estelle Russek-Cohen, Hector Izurieta, Marion Gruber, Philip Krause, Konstantin Chumakov, Jerry Weir, Hana Golding, Sheldon Morris, Gopa Raychaudhuri, and Karen Midthun.

[2] The authors alone are responsible for the views expressed in this publication, which do not necessarily represent the decisions, policy, or views of the World Health Organization or any other institution whose staff have contributed to this manuscript.

0264-410X/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2012.10.117

Contents

1. Introduction

Regulatory science is the foundation of regulatory decision-making and is used to assess the quality, safety, and efficacy of human and veterinary medicines throughout their life-span. The domains covered by regulatory science are considered to include both basic and applied biomedical sciences (such as microbiology, genetics, pharmacology, and biostatistics), clinical trial methodology and epidemiology, and social sciences (such as decision sciences, risk assessment, and communication). Regulatory science aims to contribute to the development of new tools, standards, and approaches to assess the safety, efficacy, quality, and performance of regulated products.

2. How has regulatory science contributed to improved access to vaccines?

The impact of regulatory science on improved vaccine access can be illustrated through some recent examples.

2.1. New tests for evaluation of the live-attenuated oral poliovirus vaccine (OPV): Mutant analysis by PCR and restriction endonuclease cleavage (MAPREC) and transgenic mouse tests

The OPVs that have brought the Global Polio Eradication Initiative close to success were developed by Dr. Albert Sabin by passage and testing in non-human primates. Expensive, technically demanding tests in old-world monkeys [1] were the main safety tests initially used to assure against increased virulence of the vaccine on growth for production purposes. Two outcomes of regulatory science research have revolutionized testing for revertants: (1) a molecular-based assay that used knowledge gained from studies of mutations associated with attenuation in vaccine strains; and (2) a transgenic mouse model that expresses the human poliovirus receptor, allowing viral replication and pathogenesis, similar to non-human primates and humans.

In the 1980s, major efforts were made to understand the molecular basis of attenuation, and thus neurovirulence, in poliovirus vaccines. The molecular procedure termed MAPREC was developed to measure the proportion of revertants in vaccine bulks [2] and validated through an international collaborative study, to become an official method [3] which provides a more precise assessment of vaccine batch consistency than the monkey test, and is more easily performed.

At the same time that MAPREC was being developed, the cellular receptor for poliovirus was identified [4]. The poliovirus receptor cDNA was used to prepare transgenic mice which, unlike other mice, were sensitive to poliovirus infection and developed clinical signs of infection analogous to monkeys [5]. This alternative animal model to the monkey was validated using vaccines of varying degrees of virulence comparing results to those found in monkeys [6]. A standard operating procedure was developed, and the mouse

test was accepted as a safety test for vaccines and incorporated into official WHO Recommendations [3]. Introduction of the molecular and transgenic mouse-based methods resulted in more reliable control of poliovirus vaccine consistency, improving vaccine quality and availability.

2.2. Development and use of alternative potency evaluations for release of pandemic H1N1 vaccine

In preparation for an impending influenza pandemic, investigators from the National Institutes for Food and Drug Control (NIFDC) in China and the Centre for Vaccine Evaluation, Biologics and Genetic Therapies Directorate (BGTD) of Health Canada in 2006 began jointly developing and validating new assays for vaccine quality control and lot release [7–9]. One of the aims of this collaboration was to validate assays for vaccine release in the event that international reference standards were not available for lot release using existing official methods. NIFDC led a collaborative project aimed at validating a novel quality-control assay involving deglycosylation and electrophoretic analysis of hemagglutinins (HA) from multiple manufacturing sites [7]. This alternative method for vaccine potency enabled the Chinese health authority to confidently approve the monovalent pandemic H1N1 vaccine one month earlier than would have been possible using the conventional potency assay, which requires development of antibody reagents, and helped to minimize the effects of the 2009 pandemic influenza outbreak in China. Follow-up studies confirmed that these vaccines had good safety and efficacy profiles [10].

2.3. Defining international consensus values for serological correlates of immunity for pneumococcal vaccines

The first vaccine of its kind normally undergoes full clinical protection studies for licensure, as was the case for the 7-valent pneumococcal conjugate vaccine first licensed in 2000. However, with robust biological assays to support their use, correlates and/or surrogate markers of immunity were used to licence several second-generation vaccines, thus accelerating vaccine approval and availability. WHO Recommendations for the production and control of pneumococcal conjugate vaccine [11] were developed on the basis that due to practical and ethical considerations, it would be difficult to perform protective efficacy studies on new pneumococcal conjugate vaccines and that their licensure should be based on immunogenicity studies against a licensed comparator vaccine. These Recommendations discussed the design of appropriate immunogenicity studies: (1) It was considered essential that immunogenicity studies with a new pneumococcal conjugate vaccine should provide a link to the efficacy against invasive disease that had been demonstrated for the 7-valent vaccine. (2) The immune responses to common serotypes in the new and the licensed comparator vaccine should be compared in randomized non-inferiority clinical studies. (3) The criteria for comparison should be based on serotype-specific IgG antibody concentrations measured by ELISA. (4) Measurement of functional antibody responses for a subset of vaccinated subjects using an opsonophagocytic assay (OPA) was an important additional criterion in comparing immune responses between vaccines.

Critical to this approach to licensing new pneumococcal conjugate vaccines was the need to standardize the measurement assays as well as to reach an international consensus regarding the criteria indicative of protection [12]. It is well known that biological assays have inherent variability and that those are amplified with slightly different protocols or reagents. Therefore, manufacturers, academia, and regulatory agencies sought to develop standardized assays and acceptable cut-off points used to infer protection [12]. WHO coordinated these activities, which resulted in the establishment of a WHO reference ELISA to measure IgG antibody specific for individual pneumococcal capsular polysaccharide, a quality-control sera panel for use in calibrating the ELISA, and an International Standard pneumococcal serum. Also, two WHO reference laboratories were established to facilitate the standardization of ELISA methods.

The result of these efforts was accelerated licensure and availability of second-generation pneumococcal conjugate vaccines [13,14].

2.4. Improved methods to do near real-time surveillance of health care databases facilitates safety evaluation of vaccines post-marketing

When vaccines are licensed, the clinical safety and efficacy will be based on a relatively small proportion of the population that the licensed vaccine will target. Once a licensed vaccine goes into widespread use, the inherent genetic diversity of the human population may result in AEs associated with vaccination that were not possible to see during clinical trial evaluation. In order to improve the post-market assessment of vaccine safety, the US FDA, under the Sentinel Initiative, has launched the Post-licensure Rapid Immunization Safety Monitoring (PRISM) program [15] and conducts surveillance for influenza vaccine safety in the elderly using data from Center for Medicare and Medicaid Services with novel real-time surveillance methods [16] to build on the pioneering efforts of the Centers for Disease Control and Prevention Vaccine Safety Datalink [17]. Other international efforts, such as the Vaccine Adverse Event Surveillance and Communication (VAESCO) project in Europe, create the potential for a Global Vaccine Safety Datalink. Use of near real-time surveillance not only allows for rapid identification of vaccine-related AEs, but also, in the absence of such events, helps to improve public confidence in the safety of the licensed vaccine.

3. Gaps that could be addressed by a global regulatory science agenda: Evaluating the quality of vaccines

Evaluation of vaccine quality to ensure safety and efficacy poses regulatory challenges for several reasons: (1) the complexity and diversity of the products themselves due to their biological origin; (2) the vulnerability to contamination of the biologic source materials used to manufacture vaccines (e.g., eggs, mammalian cells, fetal bovine serum, etc.); (3) many vaccines cannot withstand common purification and decontamination methods such as those used for pure chemical compounds or less complex biologics (i.e., recombinant proteins); and (4) evaluation of vaccine quality in many cases relies on a bioassay with inherent issues regarding reproducibility and robustness (e.g., animal challenge test, virus titration using cell culture, etc.).

Together, these challenges could be addressed through the following approaches: (1) development of appropriate and improved analytic methods and detection assays for infectious agents; (2) understanding the critical attributes of the vaccine that generate the protective immune response to enhance development of novel quality-control methods for vaccines; and (3) robust manufacturing processes as well as rigorous control of the source materials as part of the evaluation to ensure product quality (i.e., application of principles of Quality by Design) [18].

Some examples of advances that could be realized through a coordinated regulatory science agenda follow.

3.1. Development of new potency assays for inactivated influenza vaccines

Influenza virus changes both genetically and antigenically either by a gradual process of antigenic drift, causing epidemics, or by

sudden and dramatic antigenic shift, causing pandemics. In practice, this means that updating the vaccine is considered annually, and new, clinically relevant strains regularly replace existing vaccine strains when indicated by disease surveillance data. Efforts to streamline the process for generating updated influenza vaccines could bring significant public health benefits. One area targeted for improvement is potency testing of inactivated influenza vaccines, either by developing new methods, as was the proof-of-principle case for the monovalent pandemic H1N1 vaccine in China described above, or by improving the existing method used to measure influenza vaccine potency.

The current assay used for inactivated influenza vaccine is the single radial diffusion (SRD) assay that measures biologically relevant material, HA, in the vaccine and has been used successfully for nearly forty years. It is an immunological assay requiring reagents including antigen and antibodies which must be matched to the virus serotype used in the vaccine and thus requires updating almost every year; this is a time-consuming process.

Generation of reference antisera to HA typically involves enzymatic removal and purification of the virus HA protein, which is then used to immunize sheep. The sheep sera containing these strain-specific antibodies are collected and used as a reference standard by manufacturers in potency tests for influenza vaccines. While this approach to developing anti-HA antibodies is usually effective, there have been instances where the particular characteristics of some strains of influenza virus make it difficult to obtain sufficient amounts of HA. Therefore, an alternative approach that does not require the availability or purification of influenza virus to generate the HA immunogen has been developed. Using recombinant DNA techniques to derive plasmid DNA encoding for HA, the HA protein can be produced in vivo by direct injection of the plasmid into sheep. The level of antibody production can then be boosted by subsequent injection into the sheep of a genetically engineered viral vector encoding the same vaccine strain of HA. These sheep anti-HA antibodies have worked effectively in potency assays designed to evaluate commercially produced H1N1 and H5N1 vaccines, demonstrating the feasibility of an alternative approach to producing potency reagents [19].

In addition, availability of seasonal flu vaccine may be enhanced by successful development, evaluation, and validation of alternative potency assays. Any new assay would need to be an improvement upon current methods, measure antigenicity and monitor vaccine stability, be practical, and ideally require smaller (or no) quantities of reference standards. Methods that are being investigated include the use of reagents that cross-react with a range of different strains so they can be used in successive seasons, methods that require smaller quantities of reagents so that the production is less complex (e.g., it could abolish the current need for the industrial-scale production of antigen reagents) or the use of physico-chemical methods adapted in some way to measure only the biologically relevant conformation of the protein [20].

3.2. Research on standardization for quality control and immunogenicity of a new enterovirus 71 vaccine

Since its emergence in the United States in 1969, enterovirus 71 (EV71) has been recognized as a major public health issue across the Asia-Pacific region and beyond, causing hand-foot- and -mouth disease (HFMD), with and without neurological and systemic complications, and, in some outbreaks, high mortality. In 2008, the number of HFMD cases in mainland China amounted to 0.49 million reported cases and 126 deaths; in 2009, 1.16 million cases and 353 deaths; in 2010, 1.77 million cases and 905 deaths; and in 2011, 1.64 million cases and 506 deaths [21].

EV71 vaccines are being developed in China by more than ten manufacturers. Numerous challenges have slowed progress, including selection of vaccine strains, comparison of immunogenicity, and the lack of international and national standards as well as validated models for quality control. To overcome some of these challenges, the genetic and antigenic characteristics of different candidate vaccine strains of EV71 were studied [22], preliminary national reference standards for EV71 antigen and neutralizing antibody were established, and suitable evaluation methods for potency were devised by NIFDC [23]. In addition to these studies, the decline of maternal EV71 antibodies in infants has been investigated, identifying that the ideal time point for primary immunization for infants is around two to five months of age [24]. Consequently, today, EV71 vaccines from three manufacturers have entered phase 3 clinical trials. However, remaining challenges include developing suitable animal models so that the mechanisms of pathogenesis and protection can be determined.

3.3. Novel vaccine production technologies

Vaccine production systems in general use are well established, and include fermentation and the growth of viruses in cell culture. The use of recombinant DNA-based expression systems, such as yeast, is well established, and the regulatory issues associated with these systems have been considered in great depth over the years, with a clear product development pathway [25]. There are numerous other possible production systems, however, including expression in insect cells [26] (or living insects), in transgenic animals and plants expressing foreign proteins, or in other novel cell substrates, such as human tumor-derived cell lines. All these pose their own specific regulatory issues to be dealt with, including issues of potential tumorigenicity of residual components from the production system, or unwanted contamination from adventitious agents.

The benefit-risk evaluation for vaccines requires especially careful consideration. These are medicines usually given to healthy individuals to protect against diseases that they may never develop, even without vaccination, and typically to very young children. The acceptable level of risk and uncertainty is therefore very low, and the regulatory approach used is extremely conservative. Therefore, while novel production systems may each provide specific benefit over traditional ones, they must be evaluated carefully, using the best available science to establish an acceptable degree of confidence in the new technology.

Areas where regulatory science could impact use of novel vaccine production technologies include the following: (1) evaluating the use of transgenic plants for development of oral vaccines and whether such vaccines induce an inappropriate immune response; (2) characterizing insect cell viruses for their zoonotic potential; (3) evaluating whether a novel cell substrate would alter the antigenic phenotypes in a manner that impacts immunogenicity (either positively or negatively), such as changes in glycosylation patterns resulting from shifting from mammalian-based to insect-based, for example; and (4) determining the quantity of residual host-cell DNA that poses a risk and how to accurately measure the residual DNA.

These examples show that continued regulatory research is needed to develop new methods and understanding of the benefits and risks of new production substrates.

3.4. Development of new analytical methods

New analytical methods, especially if shown to be proxies of vaccine efficacy, may strengthen in-process control and better evaluate the quality of the finished products. Mass spectrometry, NMR spectroscopy, light scattering, and circular dichroism are useful to study structural properties, product excipients, aggregates and protein stability, and thus complement biological assays classically

used to characterize vaccines [27–29]. Additionally, they may prove useful to study vaccines derived from highly glycosylated glycoproteins, or based on bacterial capsule polysaccharides. However, careful evaluation of new methods including validation against traditional assays is required before they can be adopted for regulatory purposes.

High-throughput sequencing (also known as "Massively Parallel" or "Next Generation" sequencing) has the potential to provide very high-resolution information about all the genetic sequences present in a preparation of a vaccine, manufacturing intermediate, or raw materials used during manufacturing. Due to its higher sensitivity and greater breadth for detection of contaminating infectious agents than conventional assays [30,31], it has proven to be very useful in identifying a previously unidentified infectious agent (porcine circovirus) in a licensed rotavirus vaccine [32,33,34]. Moreover, because the method provides sequence information on a population of genomes within a preparation, rather than the most common or consensus sequence, it can also be applied to evaluate the genetic consistency of vaccines that contain nucleic acids from viruses that are prone to mutate at high rates (i.e., RNA viruses) [35].

Refinement or replacement of animal-based potency assays to allow for more robust evaluation is another area of regulatory research. The application of an ELISA-based assay to measure mouse antibodies to pertussis antigens in place of animal-based challenge assays is one such example [36]. In addition, in vitro toxin neutralization or chemical and physiochemical assays may provide useful alternatives or additional tests to traditional methods to measure potency [37,38].

4. Gaps that could be addressed by a global regulatory science agenda: Non-clinical evaluation of vaccines

4.1. Assays for novel adjuvanted vaccines

Subunit vaccines composed of recombinant or purified antigens have a good safety record but often are poorly immunogenic. The use of immunostimulatory agents such as adjuvants can enhance immune response, but safety concerns arise due to the potential for over-production of inflammatory and pyrogenic molecules. Existing non-clinical studies of adjuvanted vaccines in animal models may not always identify an increased risk for vaccine-associated AEs due to species-specific differences between the model and human population. Moreover, there is a gap in our understanding of how some adjuvants exert their immune-potentiating activities and whether the adjuvant impacts the quality of vaccine-induced protective response. Improved understanding of the adjuvant mode of action (MOA) will facilitate selection of the best adjuvant or delivery systems to achieve the desired immune responses for specific pathogens.

Another approach for additional toxicity screening tools is human cell-based assays that can predict in vivo effects of adjuvants. For example, increased levels of proinflammatory cytokines and prostaglandin E2 (PGE2) were detected in monocytes exposed to Toll-like receptor (TLR) agonists but not the approved adjuvants. When examined with an animal model, these same TLR agonists induced fever in New Zealand white rabbits that was preceded by an early peak in plasma PGE2 levels [39].

Tools also have been developed to evaluate whether adjuvants impact the quality and breadth of antibody immune responses against influenza vaccines, namely, whole genome phage display libraries (GFPDL) and surface plasmon resonance (SPR) [40]. These new tools allow measurement of the diversity, specificity, and affinity of vaccine-induced antibodies. It has been demonstrated that oil-in-water adjuvants (MF59, AS03) significantly increased the repertoire of antibody responses against pandemic influenza (H5N1; H1N1pdm09) and helped to select for high-affinity antibodies targeting the hemagglutinin globular domain (HA1) [41,42]. The increased antibody affinity correlated with improved neutralization of both homologous and heterologous influenza strains. Such cross-reactive antibodies are likely to provide better in vivo protection against influenza strains with pandemic potential.

4.2. Identification of correlates of immunity through non-clinical evaluation

In some cases, measurement of neutralizing antibodies is insufficient to evaluate the efficacy of vaccines. In particular, with those pathogens where a cell-mediated response may be critical to vaccine efficacy—such as HIV, TB, or malaria—it may be necessary to perform non-clinical studies to identify markers that correlate with protection in order to measure vaccine efficacy in clinical trials.

As one example, the development of vaccines to induce T cell-mediated immunity has been hindered by a limited understanding of the complex cellular immune responses required to protect against intracellular pathogens such as *Mycobacterium tuberculosis*. For example, specific vaccine-induced multifunctional T cell responses that correlate with protection against tuberculosis in animal models have been defined using multi-parameter flow cytometry [43]. Additionally, novel in vitro culture assays have identified cellular cytokine response profiles that are associated with the inhibition of intracellular growth by *M. tuberculosis* [44].

Exploring non-clinical methods and models such as those described above is critical to ensure that the assessment of vaccine efficacy is accurate.

5. Gaps that could be addressed by a global regulatory science agenda: Clinical evaluation of vaccines

5.1. Identification of [additional] correlates of immunity

Historically, the protective efficacy of vaccines was established through trials and epidemiological investigations which demonstrated, for many vaccines, that protection from disease correlated with levels of antibodies in the serum. For second-generation vaccines targeting established antigens, clinical trials have been simplified by measuring the specific antibody response to the vaccine. Using these immune correlates, it is also possible to assess the overall community immune status by testing blood samples from epidemiologically representative groups. Surveillance for antibodies to tetanus, diphtheria, and Japanese encephalitis vaccines are examples of this approach [45]. However, this is not possible for all vaccine types as a direct correlation between antibody levels and protective efficacy has not been shown—tuberculosis, pertussis, and HIV vaccines are examples. Moreover, the definition of "protective efficacy" used in clinical studies, (e.g., whether protection is against infection or disease, against laboratory-confirmed cases, viral load, or overt clinical disease), can also lead to different conclusions regarding efficacy. Given the complex nature of endpoints for clinical studies of different types of diseases and the vaccines used to prevent them, multiple correlates may be considered.

The complexity of evaluation of vaccine-induced immune responses can also be illustrated by current influenza vaccines. While the role of cell-mediated responses is postulated, no consensus has been reached as to how assays should be standardized to analyze these responses. In addition, vaccination against influenza by different types of vaccine (inactivated versus live attenuated), and/or a different route of administration may be associated with different protective mechanisms. Thus, a particular antibody titer may have relevance for protection for one vaccine but not others.

For example, an HI antibody titer (≥1:40) that has proved to be a protective level when induced by inactivated influenza vaccines in primed individuals cannot be used as a correlate of protection for intranasal live attenuated vaccines [46]. Furthermore, these different classes of vaccines appear to differ in their efficacy in eliciting broadly neutralizing anti-influenza antibodies, relative to the immune response following natural infection [47]. Regulatory science research projects that analyze the levels of broadly neutralizing antibodies as immune correlates could facilitate evaluation of new vaccines, adjuvants, or even the nature of the virus strains selected for use in seasonal influenza vaccines [47].

It is also recognized that for some diseases (tuberculosis, leprosy) there is a contribution from the natural immune response to the infection to the disease pathology, and these responses may complicate measurement of the specific protective immune response [48]. For viral vaccines, in particular, it has been recognized that there is a marked individual variation in the response of the vaccinee [49], and investigation of biomarkers that could predict immune response or toxicity would be of great value and facilitate development of new vaccines.

For several diseases well-controlled by vaccines, there is no active circulation of the disease-causing organism in the community as a result of successful vaccination, thus providing less effective "natural" boosting of immunity. Over time, a reservoir of susceptible individuals may build up in the community so that the disease could be re-introduced should immunization services fail to maintain a high level of coverage, as, for example, with measles or diphtheria [50]. Established immune correlates of immunity enable tracking of the possible waning immunity in a population due to lack of natural boosting and facilitates decisions about introduction of further booster immunizations (e.g., the introduction of TdaP vaccines [51]).

These examples illustrate the challenges in the development and testing of new vaccines against many diseases, as well as in tracking waning immunity following immunization. Ongoing research on correlates of protection from infection or disease is essential for regulatory authorities to evaluate the efficacy of vaccines following clinical trials as well as on an ongoing basis [52–55].

While developers of new vaccines will propose assays and parameters for evaluation of the clinical immunizing potency of their products in clinical studies, regulatory authorities need to investigate, evaluate, and collaborate to achieve international agreement on the relevance, accuracy, and sensitivity of these proposals.

5.2. Development of correlates of safety

Research on vaccines has constantly sought ways to further increase the safety of these products. Regulators have responded to such observations by implementing appropriate measures such as those described below:

- Abolishing the root cause for vaccine reactogenicity (e.g., by lowering product-and process-specific impurities below levels causing undue clinical effects).
- Providing guidance for known risk populations (e.g., as regards the use of live attenuated vaccines in immune-suppressed individuals).
- Replacing starting materials (e.g. replacing mouse brain with tissue culture for production of certain vaccines, such as JE and rabies vaccines).
- Enforcing stringent control specifications on critical starting materials, such as cells or plasma derived from human or animal origin to avoid transfer of infectious agents through vaccines.

These and other regulatory measures have been successful in minimizing side effects caused by vaccination. Developing specific and sensitive assays predictive of allergic reactions which may occur with the use of a given vaccine formulation or side effects in subpopulations known to be particularly at risk (e.g., infants with immature regulation of body temperature) will be very beneficial. Such assays are likely to be developed much faster compared to the pharmacogenomic approach outlined further below.

Unlike markers or factors suggestive of common side effects, no such tools are currently available for predicting rare and very rare adverse effects following vaccination, most of which cannot be detected even in very large clinical studies. Furthermore, very severe clinical manifestations, such as severe allergic reactions, new onset of autoimmune disorders, or even lethal outcomes such as sudden unexpected death in infancy (SUDI) or sudden infant death syndrome (SIDS) may be coincidental to, or due to an error in, administration of the vaccine, and not an inherent risk of the vaccine. For some rare side effects, a probable link has been established between vaccination and disease manifestation, such as rotavirus vaccines and intussusception. It is very unlikely, however, that these rare adverse effects are triggered by vaccination alone. Instead, vaccination may be one of several factors which, if combined, release a cascade of events ultimately resulting in an unwanted outcome. The rarity of such events suggests that some individuals may have a genetic predisposition. Since any kind of predisposition must be determined at a genomic level, identification and understanding of the significance of single nucleotide polymorphisms (SNP) or allele diversity of potential marker genes would be desirable. Pharmacogenomics is a discipline of growing importance within the field of regulatory sciences. However, unlike some chemically defined medicinal products and one monoclonal antibody (Pertuzumab "Herceptin") for which specific genetic prerequisites are required for ensuring a positive benefit/risk outcome, no specific genetic signature has been identified for predicting an increased risk of rare but severe side effects following vaccination [56].

As pharmacovigilance systems are steadily refined, the immunization community should anticipate that increased numbers of Suspected Unexpected Severe Adverse Reaction (SUSAR) will be reported for new as well as for licensed vaccines. SUSARs may also occur by chance in clinical studies. Frequently, studies are suspended or stopped whenever SUSARs are observed. Matching rare but very severe adverse reactions to individual allelic structures would both facilitate diagnosis and also communications to the public in situations where vaccines remain safe for the vast majority of a target population. However, regulatory research on identifying mechanisms of interaction between specific genetic backgrounds and an immune response following vaccination will most likely take years or even decades before usable results will become available.

5.3. Innovative clinical trial design

In recent years, innovative study designs have been proposed to speed development of promising new vaccines, where an urgent and unmet need exists. Diseases such as malaria, TB, and HIV are especially challenging.

The goals of innovative trial designs are to (1) minimize the number of ineffective candidate vaccines that proceed into Phase 2/Phase 3 trials; (2) enhance ability to identify promising candidate vaccines early; (3) more quickly obtain answers to scientific questions of interest (e.g., establishing correlates of protection); and (4) promote more efficient use of resources. The sooner non-promising vaccines can be eliminated, the more resources can be diverted to development of vaccines that most likely will be effective. For example, innovative trial designs may allow greater rigor

in Phase 2 studies and better inform the design of Phase 3 trials, thus promoting more successful outcomes.

Various types of adaptive trial designs have been considered for use with malaria, TB, and HIV vaccines [57], and stakeholders have requested regulatory advice. Recognizing this need, regulatory considerations on adaptive design trials have been published [58,59]. The US FDA views adaptive designs as those that prospectively plan for changes in the design, optimally based on blinded evaluations of accumulating data within the clinical trial. Changes to trial design based on unplanned analyses and decision paths during interim analysis, and changes based on information completely external to the trial, are not considered as adaptive designs.

Adaptive design trials demand very advanced biostatistical skills at the level of the sponsor/Principle Investigator, Data Safety Monitoring Board, and regulatory authority in order to eliminate concerns about false positives and bias. Other innovations in study designs could also be considered (e.g., enrichment studies that may target specific subpopulations at elevated risk for disease or studies that use biomarkers to deselect subjects for study if a vaccine is likely to cause an AE).

5.4. Developing mathematical models for safety data requirements across the product development lifecycle

A new regulatory approach to determine sample size in clinical trials seeks to take advantage of improvements in post-marketing safety studies to optimally allocate safety data collection at each phase of the product development lifecycle. Clinical trials of vaccines are generally larger than those for other medical products because of the very high standard of safety required for products given to very large numbers of healthy people, especially infants and children. Concern has been expressed that very large phase 3 trials, in the absence of a specific safety hypothesis, increase the time it takes to get innovative and lifesaving vaccines to people most in need [60]. While a variety of improvements have been proposed [61], a theoretically optimal framework for deciding how much data are needed at each phase of the lifecycle is lacking. Simulation of the vaccine development lifecycle has been proposed as one approach. For example, recent work linking infectious disease transmission and game theory models has allowed the systematic exploration of the interplay between disease risk and vaccine safety and effectiveness in vaccination decision-making [62]. The findings of this initial effort indicate that for vaccine-disease situations where disease risk and vaccine efficacy are sufficiently high, individuals may be more willing to tolerate greater uncertainty in vaccine safety in the early years of an immunization program, especially in the case of diseases which have no current cure. In such a situation, shifting more safety data collection to the post-marketing phase might be reasonable, assuming rigorous high quality studies can be rapidly conducted. Additional research in both the structure of the mathematical models and how to decide what constitutes the acceptable vaccine risk is needed to advance this work.

6. Gaps that could be addressed by a global regulatory science agenda: Post-marketing surveillance of vaccines

6.1. Enhancing post-marketing surveillance of vaccine safety

A lifecycle approach to safety data collection depends on the existence of systems that can rapidly conduct rigorous post-marketing vaccine safety studies to evaluate even rare AEs following immunization. However, spontaneous reporting systems, such as the US Vaccine Adverse Event Reporting System or the Uppsala Monitoring Center's Vigibase, which is part of WHO's Programme for International Drug Monitoring and involves both developed and developing countries, will also play an important role in detecting serious unexpected AEs, especially in developing countries that might not have access to large population-based electronic medical data.

Efficient and rigorous analysis of spontaneous reports of AEs following immunization remains a challenge despite improvements from the use of disproportionality data-mining methods [63]. Traditionally, spontaneous reports require evaluation by clinical experts within a "case series" framework [64] to identify unusual patterns requiring further investigation. The advent of dispropotionality data-mining methods provides the ability to summarize a large amount of information, but it is not a substitute for expert review. Case-based reasoning is a sub-field of artificial intelligence in computer science that uses a variety of algorithmic [65] and statistical [66] approaches to find reports with "similar" characteristics. Such approaches might facilitate expert identification of unexpected clinical patterns [67,68] or be used to classify reports using text mining and natural language processing [69], with resultant improvements in efficiency and timeliness.

The mining of social media for public health information has received attention recently because of the success of "Google flu trends" (http://www.google.org/flutrends/) and "HealthMaps" (http://healthmap.org/en/) in identifying infectious disease outbreaks, at least as quickly as traditional methods, but at lower cost. It is a straightforward exercise to find discussions of vaccine safety issues on internet blogs or larger services such as Twitter using standard internet search tools. Most such postings lack the necessary details that individual case safety reports submitted to spontaneous reporting systems collect, so case series evaluations that are the mainstay of current spontaneous report evaluation would likely be difficult to conduct. However, if efficient approaches to aggregating the highest quality information were developed, they might provide an earlier warning of emerging safety concerns or be especially helpful for identifying geographically localized clusters for regulators and public health authorities. Such approaches might be most effective in settings where no reporting or weak spontaneous reporting systems are present. Whether gathering such information would improve vaccine safety surveillance remains to be investigated.

Setting up spontaneous reporting systems is resource intensive, and in much of the developing world, email and internet connectivity may not allow for the mining of social media, so there might be an opportunity to leapfrog these approaches because of the wide penetration of mobile phones and other devices. Such an approach would allow health professionals to inexpensively send an alert to a central monitoring point regarding AEs. Such a project has been tried in Nigeria for monitoring use of anti-malarial drugs [70]. The collation, investigation, and analysis of such reports remain a challenge, but might be resolved by the development and deployment of artificial intelligence systems to conduct data mining and semi-automated case-series evaluations that would provide cogent summaries for human review. This would be simpler in the vaccine context, where many countries have trained vaccinating staff and a central EPI administration with an AEFI function.

7. Gaps that could be addressed by a global regulatory science agenda: Cross-cutting research

7.1. Benefit-risk methodologies

Regulatory opinions are based on balancing the desired effects or 'benefits' of a medicine against its undesired effects or 'risks'. Weighing up the benefits and risks of a medicine is a complex process, associated with some uncertainty, as the information that is available at a given point in time may be incomplete. To date, there

is no standard methodology used to aid regulatory decisions on the benefits and risks of medicines and vaccines.

Regulatory research on benefit-risk methodology aims to develop and test tools and processes for balancing multiple benefits and risks, which can be used as an aid to inform, science-based regulatory decisions about medicinal products.

A project on benefit-risk methodology is under way in the EU [71]. The first work package focuses on the current practice of benefit-risk assessment in the centralised procedure for medicinal products in the EU regulatory network [72]. The second work package examines the applicability of three frameworks and 18 quantitative approaches for assessing the benefit-risk balance [73]. It was found that multi-criteria decision analysis (MCDA) [74], an applied technology that arose from decision theory [75], can provide a theoretically sound basis for quantifying favorable and unfavorable effects, including their clinical relevance and associated uncertainties, on a common scale that shows the balance between benefits and risks. Following field-testing of MCDA, it was recognised that a complete quantitative model might not always be necessary [76]. Instead, two levels of methodology, depending on the complexity of the benefit-risk data to be assessed, may be used.

The first approach is qualitative, consisting of a table of effects and their uncertainties. This table allows simple visualisation of key effects to aid expert judgment of the benefit-risk balance. For more complex situations (e.g., multiple conflicting effects), MCDA may be a useful addition to aid the decision-making process. Also, the process of monitoring the benefit-risk balance of a medicinal product post-approval could be supported in complex or marginal cases if a quantitative model was available. As new data are received, it would be possible to update the model with the new information to see if the benefit-risk balance has changed. The last work package of the project is ongoing in the form of a pilot/training phase focused on the new methodologies. A final methodology will be agreed by the European Medicines Agency (EMA) based on the received feedback. Once finalized, it would be of interest to explore the applicability of the methodology in the context of other regulatory frameworks (outside the EU) in order to further test the usefulness of refined benefit-risk approaches in different settings.

8. Global regulatory science agenda: challenges

8.1. Updating benefit-risk analyses throughout the lifecycle of a product: Scientific and regulatory management following post-licensing discovery of signals for possible viral adventitious agents in live viral vaccines

The granting of a product license by a regulator can be considered as starting the lifecycle of a licensed vaccine, which usually lasts for several decades. Within this time period, numerous changes to the manufacturing process are likely to be introduced by manufacturers in order to apply state-of-the-art technology. These changes must be reviewed and approved by National Regulatory Authorities (NRAs). Likewise, increasing post-marketing experience will result in changes to product information provided to prescribers and patients. Depending on the number and magnitude of approved changes introduced into a manufacturing process or differences in safety and/or efficacy profiles identified by post-marketing surveillance systems compared to the pivotal safety and efficacy studies, the benefit-risk ratios may need to be reconsidered and adapted. Moreover, due to the complexity of vaccines and their respective manufacturing processes, out-of-specification batches may occasionally occur which deviate from licensed specifications and also need to be assessed individually. Such assessments need to carefully balance any major supply issues and their public health consequences caused by discarding affected batches with the potential health risks associated with the use of out-of-specification batches. In many cases, appropriate risk analyses of out-of-specification data show they do not impact the original benefit-risk ratio. When risk analysis does suggest an impact on the original benefit-risk analysis, then an affected lot is removed from the supply chain.

Recently, additional risks, undetected at the time of licensure, have been identified for vaccines (and other biologicals). In the 1990s, a laboratory found reverse transcriptase activity in licensed measles vaccines. This followed use of a new assay based on detection of reverse transcriptase activity with dramatically increased sensitivity (the PERT assay). This enzyme is characteristic of retroviruses but there are also cellular enzymes with reverse transcriptase activity. A joint effort by industry and regulatory agencies, coordinated through WHO, showed that the origin of the reverse transcriptase activity was an avian endogenous retrovirus, which is an integral part of the genome of the chicken embryo cells used to propagate the measles virus [77]. This endogenous retroviral particle was shown to be non-infectious for humans and posed no risk to vaccine recipients, so egg-based measles vaccines remained on the market [78].

More recently, massively parallel sequencing detected genomic DNA from porcine circoviruses in licensed live rotavirus vaccines (see above). Further investigations revealed that contaminated porcine trypsin used in the production process was the source of this contamination [33,34]. These laboratory data had to be complemented by use of appropriate risk-assessment tools to translate the scientific data into regulatory decisions, especially as initial rapid decision-making is essential and information was incomplete. Novel tools for assessing, quantifying, and interpreting risks associated with this type of contamination had to balance the consequences of removing an affected vaccine from the global markets, and its non-availability until the problem has been resolved, with the risks associated with continued use of the implicated product. Using such an approach, global agreement was obtained to allow the products to remain on the market [79].

8.2. Articulating the value of regulatory science in supporting global access to safe and efficacious vaccines

A global regulatory science agenda should support global access to vaccines by addressing new products, new production technologies, new analytical methods, and by mediating an increased understanding of the benefits and risks of existing and future vaccines. In addition, regulatory science helps to meet the challenges of academic and commercial vaccine development.

An example of a highly successful regulatory science project, in the framework of an international collaboration which advanced the development of a vaccine able to meet a significant public health need, is that of the MenAfriVac project. A new, safe, effective, and affordable conjugate meningitis vaccine, MenAfriVac was developed from 2001 until licensure and WHO prequalification in 2009/2010. The vaccine was based on a new, more effective conjugation method developed by two regulatory researchers in the Office of Vaccines Research and Review in FDA's Center for Biologics Evaluation and Research (CBER). This chemical method improved conjugation and simplified manufacture and purification of the vaccine's active ingredient. Early in December 2010, a vaccination campaign aimed at protecting millions of people in West Africa was launched. By the end of December 2011, about 55 million people whad been vaccinated with MenAfriVac during 2010 and 2011 [80].