viruses [5–9]. This standardization has allowed sharing of virologic surveillance data among laboratories and permitted efficient communication of this data throughout the measles control programs [10]. For molecular epidemiologic purposes, the genotype designations are considered the operational taxonomic unit, whereas related genotypes are grouped by clades. The WHO currently recognizes 8 clades, designated A, B, C, D, E, F, G, and H. Within these clades, there are 23 recognized genotypes, designated A, B1, B2, B3, C1, C2, D1, D2, D3, D4, D5, D6, D7, D8, D9, D10, E, F, G1, G2, G3, H1, and H2, and 1 provisional genotype, d11. Viruses with related sequences within some of the genotypes (eg, B3 and H1) are referred to as clusters. The WHO recommends that the 450 nucleotides coding for the COOH-terminal 150 amino acids of the nucleoprotein (N - 450) are the minimum amount of sequence data required for assigning a measles genotype [8, 11–14].

Virologic surveillance for measles was initiated in response to the global measles resurgence that occurred in the late 1980s [15]. These activities have increased significantly during the past 10 years because of the expansion of LabNet. The goal of this report is to briefly describe the lessons learned from nearly 20 years of virologic surveillance for measles, to describe the global databases for measles sequences, and to provide brief regional updates regarding circulating measles genotypes detected by surveillance activities, with a focus on the past 3 years.

LESSONS LEARNED FROM MOLECULAR EPIDEMIOLOGY OF MEASLES VIRUS

The combination of molecular epidemiology and standard case classification and reporting provides a very sensitive means to describe the transmission pathways of measles. Virologic surveillance is especially beneficial when it is possible to monitor the viral genotypes in a particular country or region over time, because this information has helped to document the interruption of transmission of endemic measles [1, 2, 16–19]. Evidence of the absence of an endemic genotype(s) is one of the criteria for verifying measles elimination in a country or region (endemic genotype(s) are defined as a genotype or genotypes associated with endemic transmission of measles).

The virologic surveillance data has shown that vaccination programs can reduce the number of co-circulating chains of transmission and eventually interrupt measles transmission. However, viruses are continually being introduced from external sources, and if the number of susceptible individuals increases, sustained transmission of the newly introduced viral genotype is possible. This results in what appears as a rapid change in the endemic genotype [20–23].

All measles vaccines belong to genotype A [24], which is a genotype that is not associated with documented endemic transmission in any part of the world. Because serologic methods cannot distinguish between a vaccine-induced antibody response and antibodies derived from natural disease, molecular characterization of viral isolates provides the only method to differentiate between natural infection and vaccine-induced adverse events. In elimination settings, rapid confirmation of these vaccine reactions will be needed to ensure that a public health response is not initiated needlessly.

Another lesson learned is that, if large measles outbreaks are occurring anywhere in the world, the viruses are soon detected almost everywhere. Measles transmission can occur anywhere, and molecular techniques are often the only method for identifying the source of an outbreak or isolated case when standard case reporting fails to identify a source. Exposures can occur in airports or other areas frequented by international travelers, such as amusement parks, conferences, and sporting events. In 2005, sequence information was used to link cases that occurred in the Netherlands to an exposure in an airport in the United States [25], whereas in 2007, sequence data were used to link cases that occurred in Texas and Michigan to an imported case at an international youth sporting event in Pennsylvania [26, 27]. Of course, molecular studies can only confirm independent sources of infection if different genotypes or clearly distinct lineages are detected (lineage is defined as a group of viruses with identical or nearly identical N-450 sequence that suggest that they represent a single chain of transmission). However, if viruses from the same lineage are detected in nonlinked cases in a particular country, the molecular data alone may not be able to differentiate between continuous circulation of virus and multiple introductions from the same source. This limitation can be addressed by analysis of the epidemiologic data. In addition, expanding the size of the region of the measles genome used for sequence comparison may allow more-precise definition of lineages, and this method is currently being evaluated by LabNet [28].

MEASLES DATABASES

Because of the importance of molecular surveillance activities for measles viruses, it is now even more necessary to be able to compare sequence information, in addition to the genotype information. Genotypes contain multiple distinct lineages. Therefore, comparing sequences is the most sensitive means to identify and map transmission chains. However, to make real-time monitoring of measles transmission chains possible, sequence and genotype information must be reported to centralized databases in a timely manner, and this information must be available to members of LabNet. Until recently, the public access database, GenBank (http://www.ncbi.nlm.gov), was the only repository for measles sequence information. Unfortunately, the release of information through GenBank is often delayed, and the entries are not curated and often lack important epidemiologic information. More recently, a number of different systems or databases have become available to collect and disseminate measles genotypic information.

However, the information collected varies widely as to whether it is aggregate or individual data, whether it includes genotype information only, and whether the sequence information is available (Table 1). The first global database available for measles genotypes was developed at WHO Headquarters in Geneva, Switzerland. Timely reporting of genotype information to the WHO database is a performance indicator for accreditation of the Regional Reference Laboratories. The WHO database for measles contained 7600 entries from 124 countries as of 1 June 2010. Although sequence data are not reported to this database, the GenBank accession number, if available, is listed. Contact details of the submitting laboratory are provided in the event that further information is needed. A similar database has been developed for rubella viruses, which contained >600 entries from 39 countries at the same point in time.

The MeaNS (Measles Nucleotide Surveillance; http://www.who-measles.org) database is a joint project between the Health Protection Agency (London, UK) and the WHO. Currently, the database collects sequence information from the complete sequence of the measles hemagglutinin (H) gene, the complete sequence of the nucleoprotein (N) gene, or the sequence of the COOH-terminal 450 nucleotides of the N gene (N-450). Additional information, including epidemiological information on the patient, is also collected. There must be sufficient information provided to create a standardized name for the sequence, as recommended by the WHO [11]. Sequence data are entered into the database either by individual contributors or by a weekly search of sequences submitted to GenBank. The data are quality checked and organized, first automatically by the database application and then manually by a curator. In addition, the deposited sequences are assigned a genotype and a cluster identifying number by matching against WHO reference sequences and the unique sequence clusters in the database, respectively. All individual sequences can be assigned for "Public viewing" or "Private." If the latter is chosen, the sequence information is only available to the administrators or those working in the WHO regional or global offices.

Dynamic reports and graphical charts can be created on any user-selected fields in the MeaNS database (eg, genotype or sequence variation in a geographical location or time period). Relevant data can be uploaded to GenBank using a specially created interface, and all submitted sequence names and genotypes are submitted weekly to the WHO. Bioinformatics tools in MeaNS allow one to find identical or similar sequences, assign a genotype, display phylogenetic trees, and to temporally and spatially track measles transmission chains. Access to the database is by registration on the website. Currently, only sequences in ASCII format can be submitted, but future developments are planned to enable uploading of sequence trace files and quality-checking mechanisms to be undertaken. It is anticipated that a similar database for rubella will be developed to enable similar tracking of rubella sequences.

To date (1 June 2010), there are 4751 sequences entered into the database from 4403 different samples. Of these, >4200 sequences are from N-450, and ~480 are full-length H gene. Thirty-four percent of the samples submitted belong to genotype D4, which reflects the recent outbreaks in different parts of the world caused by this genotype. The number of sequences submitted from different countries varies tremendously, with 39% of all the sequences in the database from the United Kingdom reflecting their use of oral fluid samples for routine measles surveillance, which can be used for both case confirmation and molecular epidemiology.

GLOBAL DISTRIBUTION OF MEASLES GENOTYPES

LabNet support for virologic surveillance is now well established in all WHO regions. Although virologic surveillance in some areas is still not adequate, a global picture has emerged (Figure 1, Tables 2–7) [2, 14]. Figure 1 is based on submissions to the WHO genotype database, whereas more-specific information for each region is presented in Tables 2–7. Some tables contain information that has not yet been reported to the WHO database. Note that the lack of an entry in the column describing endemic genotypes in Table 2 and Tables 4–7 is not meant to imply that the country has achieved measles elimination.

In general, 3 patterns of measles genotype distribution have been described. In countries that still have endemic transmission of measles, the majority of cases are caused by one or several endemic genotypes that are distributed geographically. In these cases, multiple co-circulating lineages within the endemic

Table 1. Databases That Support Molecular Epidemiology of Measles Virus

Database			Epidemiological information				Sequence	
Name	Public/private	Link	WHO name[a]	Clinical	Travel	Epi link	Genotype	Sequence
CISID	WHO	http://data.euro.who.int/cisid/	N	Y	Y	Y	N	N
WHO-LabNet	WHO-LabNet	http://workspace.who.int/sites/genotype	Y	N	Y	Y	Y	N
MeaNS	Public/private	http://www.who-measles.org	Y	Y	Y	Y	Y	Y

NOTE. WHO, World Health Organization.

[a] Standardized name as recommended by WHO.

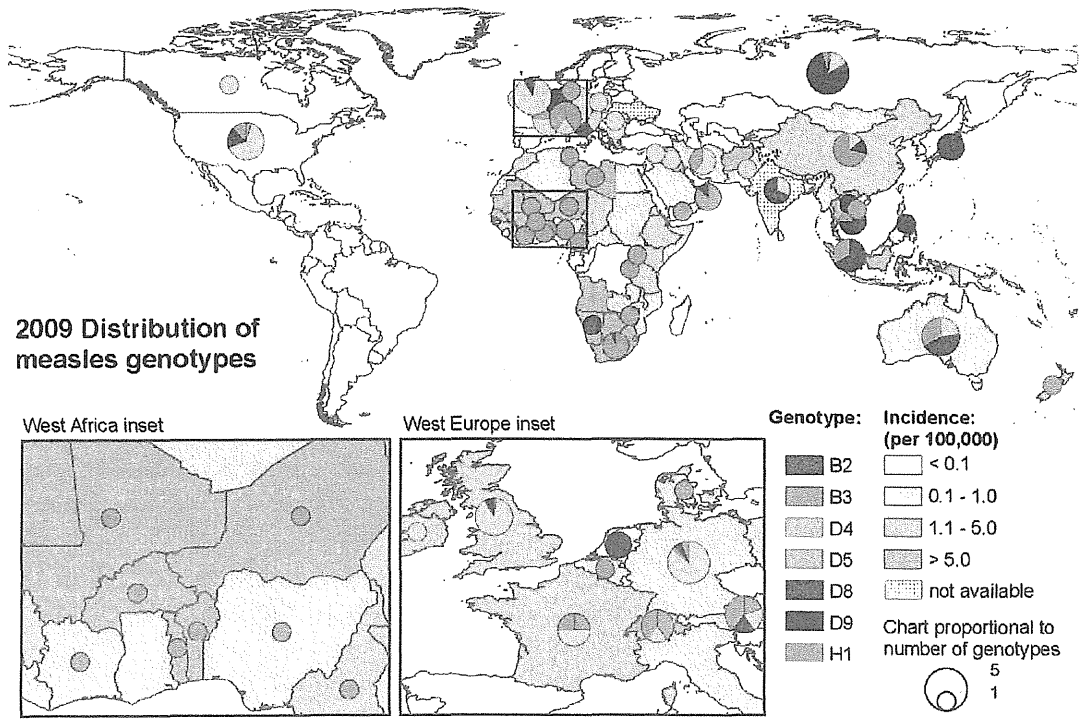

Figure 1. Global distribution of measles genotypes and measles incidence in 2009. Colored circles indicate measles genotypes reported to the World Health Organization (WHO) Database for the year 2009, and the size of the circles is proportional to the number of genotypes reported for the indicated areas (see insert of figure). For more specific information for each region, see Tables 2–7. Countries are shaded in gray to indicate measles incidence rates (see insert in figure). Two areas, Western Africa and Eastern Europe, are also shown as inserts to provide more resolution. The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the WHO concerning the legal status of any country, territory, city, or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement.

genotype or genotypes are present. In countries that have eliminated measles, the small numbers of cases are caused by a number of different genotypes that reflect various sources of imported virus and suggest the lack of sustained transmission of an endemic genotype or genotypes. The third pattern occurs in countries or regions that have had very good measles control but are experiencing an increase in the numbers of susceptible individuals because of failure to maintain high vaccination coverage rates. In this situation, reintroduction of measles usually results in outbreaks that are associated with a single genotype of virus with nearly identical sequences. In countries approaching measles elimination, introduction of measles can result in small-to-moderate outbreaks that can contribute to establishing and maintaining international chains of transmission.

MEASLES VIROLOGIC SURVEILLANCE IN THE WHO AFRICAN REGION

Virologic surveillance has improved substantially in the WHO African Region (AFR). During 2007–2009, viral genotype information was obtained from 21 countries (Table 2). The most frequently detected genotype was genotype B3, which represented 197 (89%) of 220 of the sequences reported to the WHO Global Sequence database. In 2010, genotype B3 was also found to be circulating in Malawi, Liberia, and Mauritania. Genotype B3 is clearly the endemic genotype in most of the African continent with the exception of the Northern African countries in the Eastern Mediterranean Region. Genotype B3 has been divided into 2 clusters [29]. Genotype B3, cluster 1, viruses have previously been isolated from Cameroon, Ghana, and Nigeria and as far east as Kenya and Tanzania, suggesting that genotype B3 viruses are widely distributed throughout Africa [29, 30]. The circulation of genotype B3 cluster 2 viruses appears to be more limited to Western Africa [21, 31]. Genotype B3 has been associated with frequent importations from African countries into other parts of the world [25]. Genotypes D2, D4, and D10 had been the most frequently detected genotypes in the southern and eastern parts of the African continent [32–35], although more recent outbreaks in Kenya, Uganda, Burundi, and Tanzania have been caused by genotype B3 viruses [25]. Genotypes D4 and D10, which had been circulating in eastern Africa, have not been detected in that region in the past 3 years, and D10 has not been detected anywhere since 2005 [36].

Genotype B2 was considered inactive because, until recently, no representative viruses had been isolated since 1984. However, genotype B2 viruses were detected in Angola and South Africa during 2002–2003, primarily in association with cases and importation from Angola and in the Central African Republic [37, 38], and in 2004, there was an abrupt shift from genotype B3 to genotype B2 during an outbreak in Kinshasa (Democratic Repubic of the Congo) [39]. During 2007–2009, 21 genotype B2 sequences were reported from the Democratic Republic of the Congo, Zambia, and Angola (Table 2). A single case of genotype B2 was found in Namibia in 2010, indicating that the genotype is still circulating. Two viruses in genotype D8 were detected in South Africa, and these likely represented importations of virus from India, where genotype D8 is endemic (S. Smit, unpublished data).

MEASLES VIROLOGIC SURVEILLANCE IN THE AMERICAS

The Measles and Rubella Laboratory Network for the Region of the Americas consists of 21 National Laboratories, 124 subnational laboratories, 2 Regional Reference Laboratories, and 1 Global Specialized Laboratory. Endemic transmission has been eliminated in many areas of the world, including the all of the countries in the Western Hemisphere, Region of the Americas (AMR) [16, 40]. Analysis of viruses isolated from measles cases and outbreaks in the Americas indicates that there is no ongoing transmission of an endemic genotype or genotypes. Rather, the diversity of genotypes detected in the past 15 years is indicative of multiple, imported sources of virus [17, 40]. Five different genotypes were associated with imported cases in the AMR from 2007 through 2009 (Table 3). Some were associated with isolated cases, whereas others were responsible for relatively small outbreaks [41–44]. Most recently, in 2010, an outbreak occurred in Canada that was due to importation from travelers and athletes attending the 2010 Winter Olympic Games. Genotype H1 and 2 distinct strains of D8 were detected (National Microbiology Laboratory, unpublished data).

Table 2. Measles Genotypes Detected in the African Region, 2007–2009

Country	Genotypes associated with endemic transmission	Genotypes associated with importation
Benin	B3	
DR Congo	B3, B2	
Chad	B3	
Zambia	B2, B3	
Angola	B2, B3	
Central African Republic	B3	
Cameroon	B3	
Niger	B3	
South Africa	B3	D8
Equatorial Guinea	B3	
Burkina Faso	B3	
Ethiopia	B3	
Nigeria	B3	
Burundi	B3	
Uganda	B3	
Togo	B3	
Mali	B3	
Cote d'Ivoire	B3	
Zimbabwe	B3	
Lesotho	B3	
Senegal	B3	

MEASLES VIROLOGIC SURVEILLANCE IN THE WHO EASTERN MEDITERRANEAN REGION

The WHO Regional Office for the East Mediterranean Region (EMR) has established a regional goal to eliminate measles by 2010 with laboratory support of surveillance as an essential component of the program. All 22 countries in the region have established National Measles and Rubella Laboratories.

In 2002, very few countries in the Region had initiated virologic surveillance for measles [45]. However, starting in 2007, the countries in the EMR have made remarkable progress in identifying circulating measles virus as a result of the increased capacity of the laboratory network for virus detection and genotyping [46–48].

Seventeen (77%) of the 22 countries in the EMR have identified measles genotypes between 2007 and 2009, of which 16 have reported measles genotypes to the WHO Genotype Database (Table 4). The 166 sequences reported included genotypes B3, D4, D5, D8, and H1. The most frequently detected genotype is D4, which was detected in 12 of the 17 countries and represented 53% of the sequenced genotypes. Genotype D4 has been associated with ongoing endemic transmission in the EMR and has been associated with major outbreaks in Syria, Egypt, Iraq, and Iran, despite reported high vaccination coverage in these countries. However, in some countries, such as Morocco,

Table 3. Measles Genotypes Detected in the Region of the Americas, 2007–2009

Country	Genotypes associated with imported cases
Canada	D4, D5, D8
Peru	D4
United States	B3, D4, D5, D8, H1
Argentina	D8
Jamaica	D4
Venezuela	B3
Chile	D4

Oman, and Bahrain, genotype D4 was only found in limited numbers of cases after importation from other countries that were probably within the region. Genotype B3 caused outbreaks in Libya in 2009 and was imported to Tunisia. An outbreak associated with genotype B3 occurred in Yemen and was imported to Oman. Genotype B3 was detected in 6 countries and was the second most common genotype in the region, comprising of 29% of the reported genotypes.

MEASLES VIROLOGIC SURVEILLANCE IN THE WHO EUROPEAN REGION

The WHO European Region (EUR) had adopted the target to eliminate measles and rubella and to prevent congenital rubella infection by 2015. Fifty-two of the 53 countries in the region have established National Measles and Rubella Laboratories or have access to a National Laboratory in another Member State. The EUR is very heterogeneous with respect to national strategies for measles surveillance and elimination. This is reflected in the patterns of measles transmission reported from 2007 through 2009 and in the amount of sequence information available from the different countries.

Measles cases were reported from 47 of 53 countries from 2007 through 2009, 6 countries reported zero measles cases, and 1 country did not report. Genotypes were reported from 26 countries in EUR (Table 5).

From 2007 through 2009, >2000 measles sequences were determined. Genotypes B3, D4, D5, D6, D8, D9, H1, and A (vaccine associated) were found. Transmission of the genotypes C2 and D6, which were previously endemic in some parts of Europe, has apparently been interrupted [20, 23], with no detection of C2 viruses in the EUR from 2007 through 2009 and detection of D6 only until 2007 [49]. Genotypes D4 and D5 were both associated with large numbers of cases and evidence of endemic transmission. D5 was introduced into Switzerland, presumably from Thailand, by the end of 2006. From the subsequent outbreak [50], the virus spread to Germany, Austria, Belgium, France, Norway, Denmark, and other countries [51, 52] and circulated in the EUR for at least 2.5 years. A D4 strain was introduced into the UK in spring 2007 (source unknown), spread initially within the traveler community and then within the Orthodox Jewish community before entering the more general population [53]. The outbreak continued until autumn 2009, spreading to many other countries both inside and outside the EUR. In addition, a background of other D4 variants was observed. One D4 variant was transmitted from Germany to Bulgaria and initiated an outbreak in 2009, which was ongoing in 2010 [54]. The epidemic of >24,000 cases in Bulgaria occurred after several years without endemic measles circulation and was limited mostly to the ethnic group of the Roma.

Table 4. Measles Genotypes Detected in the Eastern Mediterranean Region, 2007–2009

Country	Genotypes associated with endemic transmission	Genotypes associated with importation
Afghanistan	D4	
Bahrain	D4	D4
*Djibouti	B3	
Egypt	D4	
Iran	D4	H1
Iraq	D4	
Jordan	D4	
Kuwait	B3	D5, D8
Libya	B3	
Morocco	D8	D4
Oman	D8	B3, D4, D5
Pakistan	D4	
Qatar	D4	
Sudan	B3, D4	
Syria	D4	
Tunisia		B3
Yemen	B3	

With respect to measles elimination, the EUR gives a heterogeneous picture. Several countries in Eastern and Northern Europe reported few measles cases and have reached the elimination stage. All cases were associated with importations, and the pattern of measles genotypes detected is consistent with the elimination of indigenous measles. The Russian Federation and most Newly Independent States (NIS) countries have made remarkable progress towards elimination [49], whereas many countries in Western Europe still report a high incidence of measles. Some countries even have evidence of renewed endemic transmission after a period of low number of reported cases. Factors that contribute to the failure to reach elimination in the latter countries are low vaccination coverage in some age groups, partially because there are no mandatory vaccination programs; the presence of individuals who refuse vaccination even if offered; and the absence of effective strategies to provide vaccination to travelers and other hard-to-reach groups [55].

MEASLES VIROLOGIC SURVEILLANCE IN THE WHO SOUTHEAST ASIAN REGION

From 2007 to 2009, the majority of the 98 viral genotype reports sent to the WHO Global Measles Database from the Southeast Asian region (SEAR) were from India (Table 6). This is because of the rapid and successful expansion of LabNet activities in India, which is a country with widespread endemic circulation of measles virus. Previously, genotypes D4 and D8 have been isolated in India and Nepal [9, 56–59], and genotype D4 and D8 viruses have also been detected in measles cases imported into the United States from India [17, 40]. Genotype D7 was detected in a few sporadic cases in India [60]. During 2007–2009, all of the genotypes reported from India were D4 or D8. During this

Table 5. Measles Genotypes Detected in the European Region, 2007–2009

Country	Genotypes associated with endemic transmission	Genotypes associated with importation
Austria		D4, D5, D8, H1, B3
Belarus		D5
Belgium		D4, D5, D9, D8
Bosnia and Herzegovina		D4
Bulgaria	D4	D4, H1
Croatia		D4
Denmark		B3, D4, D5, D9, H1
France		B3, D4, D5, D8, D9, H1
Germany		B3, D4, D5, D8, D9, H1
Israel		D4, D9
Kazakhstan	D6	
Kyrgyzstan		D4, D6
Netherlands		B3, D4, D5, D8, D9
Norway		D5
Poland		D4, D6
Portugal		D4
Republic of Moldova		D6
Romania		D4, D5
Russian Federation	D6	B3, D4, D5, D6, D8, D9, H1
Serbia		D4, D9
Spain		D4, D5, D9
Switzerland	D5	B3, D4
The former Yugoslav Republic of Macedonia		D4
Turkey		D4
Ukraine	D6	
United Kingdom of Great Britain and Northern Ireland	D4	B3, D4, D5, D8, D9
Uzbekistan	D6	

period, genotypes D5 and D9 were detected in Thailand, and genotypes D4 and D8 were detected in Nepal. In 2007, genotype H1 was detected in the Democratic People's Republic of Korea.

Virologic surveillance prior to 2007 also indicated that genotypes G2, G3, and D9 appeared to be the endemic genotypes in Indonesia and East Timor [61]. Genotype G2 has been detected in Thailand in the early 2000s, in addition to genotype D5. Genotype D5 was detected from a small outbreak in the Maldives in 2005, and genotypes D5 and D9 were detected in Myanmar.

Table 6. Measles Genotypes Detected in the Southeast Asian Region, 2007–2009

Country	Genotypes associated with endemic transmission	Genotypes associated with importation
India	D4, D8	
Bangladesh	D4, D8	
Maldives	D5	
Myanmar	D5, D9, d11[a]	
Thailand	D5, D9, G2	
Indonesia	G2, G3, D9	
Nepal	D4, D8	
DRPK	H1	

NOTE. [a] Detected in China

MEASLES VIROLOGIC SURVEILLANCE IN THE WHO WESTERN PACIFIC REGION

With the target to eliminate measles by 2012, all of the countries in the Western Pacific Region (WPR) are conducting case-based surveillance for measles, and virologic surveillance is well established in the region. Some countries (eg, Australia) have a pattern of viral genotypes that is consistent with elimination of endemic virus (Table 7). From 2007 through 2009, countries in WPR submitted genotype information from 1127 cases to the WHO Global Measles Database. Of these, 990 were genotype H1, and 820 of these reports were from China (Table 7). Therefore, genotype H1 continues to be the indigenous strain in China. The Chinese genotype H1 sequences have been divided into 3 clusters, H1a, H1b, and H1c [62], and all of the recent sequences were members of cluster H1a [63, 64]. The H1

Table 7. Measles Genotypes Detected in the Western Pacific Region, 2007–2009

Country	Genotypes associated with endemic transmission	Genotypes associated with importation
Australia		D4, D8, H1, D9, D5
Cambodia	D9	H1
China	H1	D4, D9, d11
China, Hong Kong SAR	H1	H1, D9
China, Macao SAR		D9, H1
Japan	D5, H1	D4, D8, D9
Lao People's Democratic Republic	H1	D9
Malaysia	D9, G3	
New Zealand		B3, H1, D4
Philippines	D9, G3	
Republic of Korea	H1	B3, D5
Singapore	D9	D4, D5, D8, D9, H1
Vietnam	H1	

genotype is also endemic in Viet Nam and caused outbreaks in 2009. In addition to the indigenous viruses in China, genotypes D4 and D9 were detected in association with imported cases in 2009 [65]. A new genotype, designated as provisional genotype d11, was detected in 2009 in viruses that were imported into China from Myanmar [66].

Elsewhere in WPR, genotypes H1, D9, G3, and D5 were associated with endemic transmission in several countries, and genotypes D4, D5, D8, D9, H1, and B3 were associated with imported cases in the region (Table 7). Genotype D3 had been associated with endemic transmission in the Philippines, but recent viral isolates are genotypes D9 and G3. Genotype D3 has not been detected in the WPR or in any other region since the mid-2000s. During 2007–2009, 13 (87%) of the 15 countries in the WPR (excluding the 20 Pacific Island countries) have identified measles genotypes.

SUMMARY AND FUTURE CHALLENGES

As several regions move toward measles elimination goals, adequate virologic surveillance will become an essential component of the surveillance systems that will be needed to verify that the elimination targets have been reached. The information presented in this report has briefly documented the tremendous expansion of global virologic surveillance for measles through the actions of LabNet. The laboratory methods to perform genetic analysis of wild-type measles strains are firmly established in all regions, and all of the WHO Regional Reference Laboratories have the capacity for virus isolation, reverse-transcription polymerase chain reaction (RT-PCR), and sequencing. An efficient mechanism has been established for timely reporting of sequence information. All of the LabNet laboratories are now using a single cell line, Vero/hSLAM [67], for isolation of both measles and rubella viruses. It is particularly encouraging that some countries were able to detect very small numbers of imported cases despite widespread circulation of an endemic genotype.

Although viral isolates were not obtained from all countries during the 3-year time period presented in this report, most countries with endemic measles have conducted some baseline virologic surveillance. One of the challenges for LabNet will be to continue to expand virologic surveillance activities, especially in those countries where no or only sporadic virologic surveillance has occurred. Periodic training is essential to ensure that laboratory staff are proficient in the methods for cell culture as well as RT-PCR and sequencing, if applicable. LabNet laboratories are now actively developing quality control and quality assurance protocols for the molecular techniques so that this part of laboratory surveillance will be held to the same high standards that are currently in place for serologic testing.

The other major challenge for virologic surveillance is obtaining adequate samples from representative cases and outbreaks. This is particularly challenging in areas that lack the infrastructure for sample collection, storage, and shipment. The use of alternative sample collection methods, such as oral fluids and blood dried onto filter paper, should help in this regard [68].

Molecular surveillance in many countries and in the EUR, in particular, has shown that the endemic genotype or genotypes can change relatively quickly. Therefore, constant monitoring of cases and outbreaks is necessary.

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References

1. Rota PA, Bellini WJ. Update on the global distribution of genotypes of wild type measles viruses. J Infect Dis **2003**; 187(Suppl 1): S270–S276.
2. Rota PA, Featherstone DA, Bellini WJ. Molecular epidemiology of measles virus. Curr Top Microbiol Immunol **2009**; 330:129–150.
3. Global measles and rubella laboratory network–update. Wkly Epidemiol Rec **2005**; 80:384–388.
4. Global measles and rubella laboratory network, January 2004–June 2005. MMWR Morb Mortal Wkly Rep **2005**; 54:1100–1104.
5. World Health Organization. Standardization of the nomenclature for describing the genetic characteristics of wild-type measles viruses. Wkly Epidemiological Rec **1998**; 73:265–272.
6. Standardization of the nomenclature for genetic characteristics of wild-type rubella viruses. Wkly Epidemiol Rec **2005**; 80:126–132.
7. Update of standard nomenclature for wild-type rubella viruses, 2007. Wkly Epidemiol Rec **2007**; 82:216–222.
8. Nomenclature for describing the genetic characteristics of wild-type measles viruses (update). Wkly Epidemiol Rec **2001**; 76:249–251.
9. Nomenclature for describing the genetic characteristics of wild-type measles viruses (update). Part I Wkly Epidemiol Rec **2001**; 76:242–247.
10. World Health Organization (WHO). Manual for the diagnosis of measles and rubella infection, 2nd ed. Geneva, Switzerland: WHO, **2007**.
11. Expanded Programme on Immunization (EPI). Standardization of the nomenclature for describing the genetic characteristics of wild-type measles viruses. Wkly Epidemiol Rec **1998**; 73:265–269.
12. Update of the nomenclature for describing the genetic characteristics of wild-type measles viruses: new genotypes and reference strains. Wkly Epidemiol Rec **2003**; 78:229–232.
13. New genotype of measles virus and update on global distribution of measles genotypes. Wkly Epidemiol Rec **2005**; 80:347–351.
14. Global distribution of measles and rubella genotypes–update. Wkly Epidemiol Rec **2006**; 81:474–479.
15. Rota JS, Hummel KB, Rota PA, Bellini WJ. Genetic variability of the glycoprotein genes of current wild-type measles isolates. Virology **1992**; 188:135–142.
16. Rota JS, Heath JL, Rota PA, et al. Molecular epidemiology of measles virus: identification of pathways of transmission and implications for measles elimination. J Infect Dis **1996**; 173:32–37.
17. Rota PA, Liffick SL, Rota JS, et al. Molecular epidemiology of measles viruses in the United States, 1997–2001. Emerg Infect Dis **2002**; 8: 902–908.
18. Mulders MN, Truong AT, Muller CP. Monitoring of measles elimination using molecular epidemiology. Vaccine **2001**; 19:2245–2249.
19. Riddell MA, Rota JS, Rota PA. Review of the temporal and geographical distribution of measles virus genotypes in the prevaccine and post-vaccine eras. Virol J **2005**; 2:87.
20. Kremer JR, Brown KE, Jin L, et al. High genetic diversity of measles virus, world health Organization European region, 2005–2006. Emerg Infect Dis **2008**; 14:107–114.
21. Mulders MN, Nebie YK, Fack F, et al. Limited diversity of measles field isolates after a national immunization day in Burkina Faso: progress from endemic to epidemic transmission? J Infect Dis **2003**; 187(Suppl 1):S277–S282.
22. Rima BK, Earle JA, Yeo RP, et al. Temporal and geographical distribution of measles virus genotypes. J Gen Virol **1995**; 76: 1173–1180.
23. Santibanez S, Tischer A, Heider A, Siedler A, Hengel H. Rapid replacement of endemic measles virus genotypes. J Gen Virol **2002**; 83:2699–2708.
24. Rota JS, Wang ZD, Rota PA, Bellini WJ. Comparison of sequences of the H, F, and N coding genes of measles virus vaccine strains. Virus Res **1994**; 31:317–330.
25. Rota J, Lowe L, Rota P, et al. Identical genotype B3 sequences from measles patients in 4 countries, 2005. Emerg Infect Dis **2006**; 12:1779–1781.
26. Multistate measles outbreak associated with an international youth sporting event–Pennsylvania, Michigan, and Texas, August–September 2007. MMWR Morb Mortal Wkly Rep **2008**; 57:169–173.
27. Chen TH, Kutty P, Lowe LE, et al. Measles outbreak associated with an international youth sporting event in the United States. Pediatr Infect Dis J **2007**; 29:794–800.
28. Bankamp B, Lopareva EN, Kremer JR, et al. Genetic variability and mRNA editing frequencies of the phosphoprotein genes of wild-type measles viruses. Virus Res **2008**; 135:298–306.
29. Hanses F, Truong AT, Ammerlaan W, et al. Molecular epidemiology of Nigerian and Ghanaian measles virus isolates reveals a genotype circulating widely in western and central Africa. J Gen Virol **1999**; 80:871–877.
30. El Mubarak HS, van de Bildt MW, Mustafa OA, et al. Genetic characterization of wild-type measles viruses circulating in suburban Khartoum, 1997–2000. J Gen Virol **2002**; 83:1437–1443.
31. Kouomou DW, Nerrienet E, Mfoupouendoun J, Tene G, Whittle H, Wild TF. Measles virus strains circulating in Central and West Africa: geographical distribution of two B3 genotypes. J Med Virol **2002**; 68:433–440.
32. Kreis S, Vardas E, Whistler T. Sequence analysis of the nucleocapsid gene of measles virus isolates from South Africa identifies a new genotype. J Gen Virol **1997**; 78:1581–1587.
33. Nigatu W, Jin L, Cohen BJ, et al. Measles virus strains circulating in Ethiopia in 1998-1999: molecular characterisation using oral fluid samples and identification of a new genotype. J Med Virol **2001**; 65:373–380.
34. Mbugua FM, Okoth FA, Gray M, et al. Molecular epidemiology of measles virus in Kenya. J Med Virol **2003**; 71:599–604.
35. Muwonge A, Nanyunja M, Rota PA, et al. New measles genotype, Uganda. Emerg Infect Dis **2005**; 11:1522–1526.
36. Baliraine FN, Bwogi J, Bukenya H, et al. Possible interruption of measles virus transmission, Uganda, 2006–2009. Emerg Infect Dis **2011**; 17:110–113.
37. Smit SB, Hardie D, Tiemessen CT. Measles virus genotype B2 is not inactive: evidence of continued circulation in Africa. J Med Virol **2005**; 77:550–557.
38. Gouandjika-Vasilache I, Waku-Kouomou D, Menard D, et al. Cocirculation of measles virus genotype B2 and B3.1 in Central African Republic during the 2000 measles epidemic. J Med Virol **2006**; 78:964–970.
39. Kremer JR, Nkwembe E, Bola Oyefolu AO, et al. Measles virus strain diversity, Nigeria and democratic republic of the congo. Emerg Infect Dis **2010**; 16:1724–1730.
40. Rota JS, Rota PA, Redd SB, Redd SC, Pattamadilok S, Bellini WJ. Genetic analysis of measles viruses isolated in the United States, 1995–1996. J Infect Dis **1998**; 177:204–208.
41. Update: measles–United States, January–July 2008. MMWR Morb Mortal Wkly Rep **2008**; 57:893–896.
42. Measles–United States, January 1–April 25, 2008. MMWR Morb Mortal Wkly Rep **2008**; 57:494–498.
43. Outbreak of measles–San Diego, California, January–February 2008. MMWR Morb Mortal Wkly Rep **2008**; 57:203–206.
44. Measles among adults associated with adoption of children in China–California, Missouri, and Washington, July–August 2006. MMWR Morb Mortal Wkly Rep **2007**; 56:144–146.
45. Alla A, Liffick SL, Newton BR, Elaouad R, Rota PA, Bellini WJ. Genetic analysis of measles viruses isolated in Morocco. J Med Virol **2002**; 68:441–444.
46. Alla A, Waku-Kouomou D, Benjouad A, Elaouad R, Wild TF. Rapid diversification of measles virus genotypes circulating in Morocco during 2004–2005 epidemics. J Med Virol **2006**; 78:1465–1472.
47. Djebbi A, Bahri O, Mokhtariazad T, et al. Identification of measles virus genotypes from recent outbreaks in countries from the Eastern Mediterranean Region. J Clin Virol **2005**; 34:1–6.
48. Haddad-Boubaker S, Rezq M, Smeo MN, et al. Genetic characterization of clade B measles viruses isolated in Tunisia and Libya 2002–2009

and a proposed new subtype within the B3 genotype. Virus Res **2010**; 153:258–264.
49. Shulga SV, Rota PA, Kremer JR, et al. Genetic variability of wild-type measles viruses, circulating in the Russian Federation during the implementation of the National Measles Elimination Program, 2003–2007. Clin Microbiol Infect **2009**; 15:528–537.
50. Richard JL, Masserey Spicher V. Large measles epidemic in Switzerland from 2006 to 2009: consequences for the elimination of measles in Europe. Euro Surveill **2009**; 14.
51. Schmid D, Holzmann H, Schwarz K, et al. Measles outbreak linked to a minority group in Austria, 2008. Epidemiol Infect **2010**; 138:415–425.
52. Waku-Kouomou D, Freymuth F, du Chatelet IP, Wild TF, Horvat B. Co-circulation of multiple measles virus genotypes during an epidemic in France in 2008. J Med Virol **2010**; 82:1033–1043.
53. Cohuet S, Bukasa A, Heathcock R, et al. A measles outbreak in the Irish traveller ethnic group after attending a funeral in England, March–June 2007. Epidemiol Infect **2009**; 137:1759–1765.
54. Marinova L, Kojouharova M, Mihneva Z. An ongoing measles outbreak in Bulgaria, 2009. Euro Surveill **2009**; 14:1–3.
55. Kremer JR, Muller CP. Measles in Europe–there is room for improvement. Lancet **2009**; 373:356–358.
56. Truong AT, Mulders MN, Gautam DC, et al. Genetic analysis of Asian measles virus strains–new endemic genotype in Nepal. Virus Res **2001**; 76:71–78.
57. Wairagkar N, Rota PA, Liffick S, Shaikh N, Padbidri VS, Bellini WJ. Characterization of measles sequences from Pune, India. J Med Virol **2002**; 68:611–614.
58. Mohan A, Murhekar MV, Wairgkar NS, Hutin YJ, Gupte MD. Measles transmission following the tsunami in a population with a high one-dose vaccination coverage, Tamil Nadu, India 2004–2005. BMC Infect Dis **2006**; 6:143.
59. Ramamurty N, Raja D, Gunasekaran P, Varalakshmi E, Mohana S, Jin L. Investigation of measles and rubella outbreaks in Tamil Nadu, India-2003. J Med Virol **2006**; 78:508–513.
60. Vaidya SR, Wairagkar NS, Raja D, et al. First detection of measles genotype D7 from India. Virus Genes **2008**; 36:31–34.
61. Chibo D, Riddell M, Catton M, Birch C. Novel measles virus genotype, East Timor and Australia. Emerg Infect Dis **2002**; 8:735–737.
62. Zhang Y, Zhu Z, Rota PA, et al. Molecular epidemiology of measles viruses in China, 1995–2003. Virol J **2007**; 4:14.
63. Zhang Y, Ji YX, Zhu Z. [Circulating pattern analysis for endemic measles viruses in mainland of China]. Zhongguo Yi Miao He Mian Yi **2009**; 15:97–103.
64. Zhang Y, Ji Y, Jiang X, et al. Genetic characterization of measles viruses in China, 2004. Virol J **2008**; 5:120.
65. Zhang Y, He JL, Sun L. [D9 measles virus was first isolated from an imported measles case in Sichuan Province of China]. Zhongguo Yi Miao He Mian Yi **2009**; 15:304–309.
66. Zhang Y, Ding Z, Wang H, et al. New measles virus genotype associated with outbreak, China. Emerg Infect Dis **2010**; 16:943–947.
67. Ono N, Tatsuo H, Hidaka Y, Aoki T, Minagawa H, Yanagi Y. Measles viruses on throat swabs from measles patients use signaling lymphocytic activation molecule (CDw150) but not CD46 as a cellular receptor. J Virol **2001**; 75:4399–4401.
68. Recommendations from an ad hoc Meeting of the WHO Measles and Rubella Laboratory Network (LabNet) on use of alternative diagnostic samples for measles and rubella surveillance. MMWR Morb Mortal Wkly Rep **2008**; 57:657–660.

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Amino Acid Substitutions in Matrix, Fusion and Hemagglutinin Proteins of Wild Measles Virus for Adaptation to Vero Cells

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Key Words
CD46 · Fusion protein · Hemagglutinin protein · Matrix protein · Measles virus · SLAM

Abstract
Background: Wild-type measles virus (MV) is isolated in B95a but not in Vero cells. Through an adaptation process of wild-type MV to Vero cells, several amino acid substitutions were reported. ***Methods:*** Six strains were adapted to Vero cells and membrane (M), fusion (F) and hemagglutinin (H) genes were sequenced. Cell fusion was assessed and recombinant MVs were constructed, having wild-type H or M gene with or without mutations. ***Results:*** No F gene substitution was noted. Amino-acid substitutions at positions 481 from Asn to Tyr (N481Y) and 546 from Ser to Gly (S546G) were observed in the H protein. Glu at position 89 of the M protein was substituted for Gly (E89G) and two mutations were noted at positions 62 (S62R) and 83 (S83P) in M protein. Recombinant viruses with mutation(s) detected in Vero-adapted strains induced a cytopathic effect and grew well in Vero cells, but those with the wild type did not. Recombinant viruses with mutation(s) demonstrated lower viral growth in B95a cells. ***Conclusions:*** Substitutions of E89G, S62R and S83P of the M protein were newly observed through adaptation to Vero cells, besides the mutations described in previous reports, with varying adaptation for each strain.

Introduction

Measles virus (MV) is a member of the genus *Morbillivirus*, family Paramyxoviridae, order Mononegavirales, characterized by non-segmented, negative-strand RNA virus. The genome consists of 15,894 nucleotides, coding six structural proteins: nucleoprotein (N), phospho (P), membrane (M), fusion (F), hemagglutinin (H) and large (L) proteins. The genomic RNA is encapsidated with N protein and consisted of ribonucleoprotein, together with RNA-dependent RNA polymerase complex of L and P proteins [1, 2]. MV has two envelop glycoproteins, F and H, which play an important role in virus attachment and subsequent virus cell fusion as well as cell-to-cell fusion [3–5]. The Edmonston strain was isolated in 1954 using a primary culture of human kidney cells and, thereafter, MV was isolated using Vero cells, but the sensitivity of Vero cells was poor, and two or three blind passages were required. MV was isolated more efficiently in B95a cells, marmoset lymphoblastic cell lines transformed by Epstein-Barr virus [6]. CD46 was initially reported to act as a cellular receptor for laboratory-adapted, vaccine strains of MV. CD46 is a member of the regulators of the complement activation gene cluster and is widely expressed on epithelial cells, but not on lymphocytic cells [7, 8]. In 2000, human signaling lymphocyte activation molecule (SLAM; CD150) was reported as the receptor of wild-type MV and it was expressed on lymphocytic cells [9, 10]. The

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Edmonston strain and relevant vaccine strains were found to use both SLAM and CD46 as receptors and circulating wild-type strains utilize SLAM as a receptor, but not CD46 [9, 10].

Several amino-acid changes in H, M, L and P proteins and/or accessory V and C proteins were responsible for attenuation through adaptation to Vero cells. Some authors reported that the majority of MV strains using CD46 as a receptor have tyrosine at position 481, whereas wild-type strains have asparagine at this position [11–17]. When the wild-type MV strains became adapted to grow in Vero cells, the substitution at position 481 of H protein from asparagine to tyrosine (N481Y) was often observed after several passages [16, 18]. In some Vero cell-adapted strains, a substitution at position 546 of the H protein from serine to glycine (S546G) was observed instead of the N481Y substitution [19–22]. A single substitution of N481Y or S546G enabled the H protein of wild-type MV strains to utilize CD46, without influencing their ability to use SLAM. In addition, two amino-acid differences were observed in Edmonston-derived strains in comparison with wild-type strains at positions 64 and 89 of M protein (P64S and E89K), which allowed an interaction of M protein with the cytoplasmic tail of H protein, thereby enhancing cell fusion and assembly of infectious particles in Vero cells [23–25].

In this report, amino-acid substitutions were investigated in M, F and H proteins of Vero-adapted strains from six wild-type MV strains isolated in B95a cells, in comparison with the original wild types. Mutations of N481Y or S546G of the H protein region were observed as previously reported. In addition, mutations in S62R, S83P and E89G of the M protein region were noted, being different from the mutations described in previous reports. Recombinant MV strains with mutations in the H and M genes were constructed to conduct a functional analysis of the mutations.

Materials and Methods

Cells and Viruses

Six strains of the wild type were used in this study.

MVi/Tokyo.JPN/17.07-AN/B4, MVi/Mie.JPN/19.07-OY/B4, MVi/Mie.JPN/23.07-TY/B3, MVi/Mie.JPN/41.07-MA/B3 and MVi/Mie.JPN/03.08-KU/B4 were genotype D5 isolated in 2007/2008 outbreaks in Japan, using B95a cells after three or four passages. MVi/Aichi.JPN/44.06/B3 was genotype D9 [26]. Through several passages in Vero cells, eight Vero-adapted strains were obtained. AN-V4 was obtained after four passages of MVi/Tokyo.JPN/17.07-AN/B4 in Vero cells. OY-V4 and OY-V22 were obtained after four and 22 passages of MVi/Mie.JPN/19.07-OY/B4 in Vero cells, respectively. TY-V4, TY-V22, MA-V15, KU-V4 and D9-V4 strains were obtained after passages of respective strains in Vero cells. MVAT7 pol., non-replicative vaccinia virus expressing T7 RNA polymerase (a kind gift from Dr. G. Sutter), was used for fusion analysis and the recovery of infectious viruses.

B95a cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), and Vero cells in MEM supplemented with 5% FCS. 293 T cells were cultured in MEM supplemented with 10% FCS.

Construction of F and H Expression Plasmids and Fusion Experiment

The H protein expression plasmids were constructed from Vero-adapted strains (AN-V4, OY-V4, OY-V22, TY-V22, MA-V15, KU-V4 and D9-V4 strains) and their original wild-type isolates and AIK-C strain. The H gene was amplified by RT-PCR using the set of primers H-ATG (5′-GTTGAATTCATGTCACCAC-AACGAGACCGGA-3′) and H-TAG (5′-AATGCGGCCGCCT-ATCTGCGATTGGTTCCA-3′), containing restriction enzyme sites of *Eco*RI and *Not*I (underlined). The amplified DNA fragments were cloned into multicloning sites located downstream of the T7 promoter of pBluescript SK II–. Several clones were sequenced to analyze the frequency of mutations. Constructed H expression plasmid (0.2 μg) was co-transfected together with the AIK-C F expression plasmid into a monolayer of B95a or Vero cells infected with recombinant vaccinia virus, MVAT7 pol., using Mirus Superfect III (Invitrogen Life Technologies, Carlsbad, Calif., USA) [27]. Cells were fixed with cooled acetone and further subjected to indirect immunofluorescent (IF) staining.

Construction of Recombinant MV Strains with Mutations

The infectious recombinant MV strains were recovered from the infectious cDNA clone based on the AIK-C measles vaccine strain, expressing wild-type measles H protein cloned from the wild-type MV [27, 28], and the recombinant infectious cDNA constructions are shown in figure 1. Briefly, full-length cDNA of the AIK-C strain was divided into two parts: the first half consisted of AIK-C cDNA from the leader to the *Pac*I site at nucleotide position 7238 of the AIK-C genome, and the second half of the AIK-C cDNA consisted of H and L regions from the 7239 *Pac*I site to the trailer sequence. The *Asc*I site (GGCGCGCC) was artificially introduced by adding a GGCGCG sequence upstream of the genome position 3433 in the P/M junction, and R1 and R2 sequences were added. The green fluorescent protein (GFP) sequence was inserted using *Nco*I and *Not*I restriction enzyme sites in accordance with the rule of six of the genome length [29, 30], designated as pMVAIK P/M-GFP. The H gene of the wild-type or Vero-adapted strains was cloned at *Nhe*I (genome position 7426) and *Pvu*II (genome position 9082) of the second half of the AIK-C cDNA and combined with the first half of the cDNA. The M gene of the wild-type or Vero-adapted strains was cloned at *Bgl*II (genome position 3445) and *Ban*III (genome position 4312) sites of the first half of the AIK-C cDNA clone and combined with the second half to construct full-length cDNA.

For the recovery of the recombinant MV, 293 T cells were infected with MVAT7 pol., and then 0.5 μg of pAIK-N, 0.25 μg of pAIK-P, 0.1 μg of pAIK-L and 1.5 μg of full-length recombinant cDNA, using Mirus Superfect III (Invitrogen Life Technologies) modified from previous reports [27, 28]. After 2 days of culture,

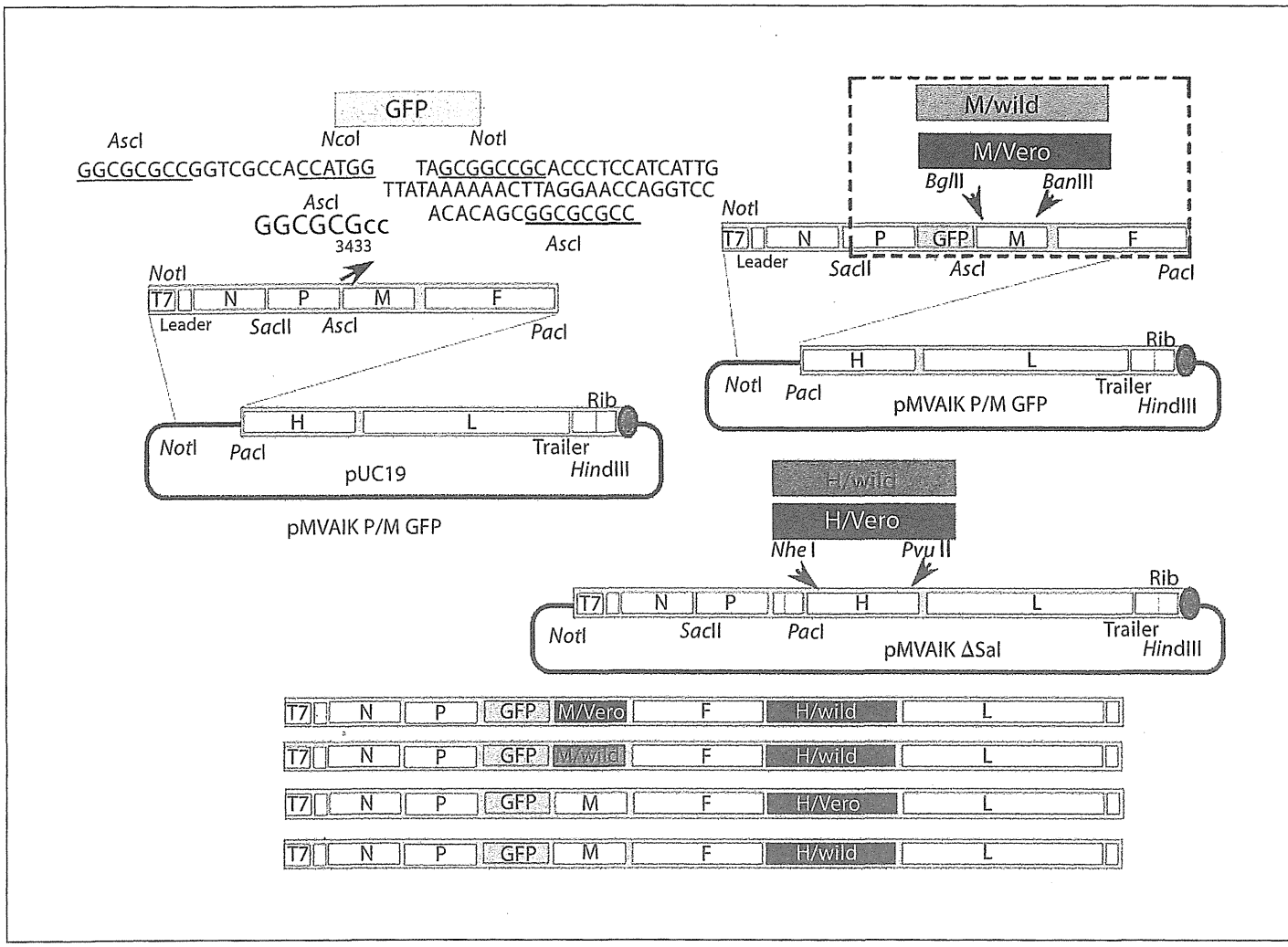

Fig. 1. Construction of recombinant MV. Full-length AIK-C cDNA was divided into two parts at the *Pac*I restriction enzyme site. The H genes of the wild-type and Vero-adapted strains were amplified and cloned using *Nhe*I and *Pvu*II restriction enzyme sites. The M gene was inserted at *Bgl*II and *Ban*III restriction enzyme sites. The *Asc*I site (GGCGCGCC) was artificially introduced by adding a GGCGCG sequence upstream of the genome position 3433 in the P/M junction, and R1 and R2 sequences were added. The GFP sequence was inserted at the P/M junction.

293 T cells were co-cultured with B95a cells. Infectious virus particles were rescued through two blind passages in B95a cells at 32.5° in 5% CO_2.

Sequence Analysis

The M, F and H genes of the wild-type isolates and Vero-adapted strains were amplified by RT-PCR and sequenced by dye terminator methods using ABI 3130 (Applied Bio Systems Japan, Tokyo). Consensus sequence was defined as the dominant sequence determined by direct sequence and respective gene cloning.

Indirect IF Staining and GFP Expression

B95a or Vero cells were cultured in 8-well LabTek Glass slides (Nalge Nunc International, Rochester, N.Y., USA) and infected with recombinant MV strains. GFP expression was confirmed and cells were fixed with cooled acetone and further subjected to indirect IF staining using 1:100 dilution of monoclonal antibody against measles H protein (kindly supplied by Dr. T. A. Sato, National Institutes for Infectious Diseases) and that against N protein (Chemicon, Temecula, Calif., USA). They were stained with 1:100 dilution of anti-mouse IgG monoclonal antibody labeled with FITC (Sigma-Aldrich, St. Louis, Mo., USA).

Virus Growth and GFP Expression

B95a or Vero cells were infected with recombinant MV strains at MOI = 0.01 and culture supernatant was obtained on days 1, 3, 5 and 7 of culture. Virus infectivity was calculated by the Reed-Muench method using B95a cells, and GFP expression was monitored with a microplate fluorescent reader, FLx 800 (Bio-Tek Instruments, Winooski, Vt., USA).

Table 1. Amino-acid substitutions of wild-type isolates after adaptation to Vero cells

MV strains	Genotype	Passage in Vero cells	H original		M original		
			481Asn	546Ser	62Ser	83Ser	89Glu
MVi/Tokyo.JPN/17.07-AN/B4	D5	AN-V4	–[1]	Gly	–	–	–
MVi/Mie.JPN/19.07-OY/B4	D5	OY-V4	Tyr	–	–	–	–
MVi/Mie.JPN/19.07-OY/B4	D5	OY-V22	Tyr	Gly	Arg	Pro	–
MVi/Mie.JPN/23.07-TY/B3	D5	TY-V4	–	–	–	–	–
MVi/Mie.JPN/23.07-TY/B3	D5	TY-V22	–	Gly	–	–	–
MVi/Mie.JPN/41.07-MA/B3	D5	MA-V15	Tyr	–	–	–	–
MVi/Mie.JPN/03.08-KU/B4	D5	KU-V4	–	Gly	–	–	–
MVi/Aichi.JPN/44.06/B3	D9	D9-V4	–	–	–	–	Gly

AN-V4 was obtained after four passages of MV/Tokyo.JPN/17.07-AN/B4 in Vero cells. H original/M original = Amino acids of the original strain.

[1] No amino-acid substitution after adaptation to Vero cells.

Table 2. Sequence diversity of M and H protein-encoding plasmids derived from Vero-adapted virus strains

	H gene				M gene				
	N481Y	S546G	481/546	H-wt	S62R	S83P	62/83	E89G	M-wt
OY-V4	4	0	0	6					
OY-V22	3	6	1	2	2	0	22		0
TY-V22	0	6	0	1					
MA-V15	6	0	0	0					
KU-V14	0	7	0	0					
D9-V4								4	2

Results

Amino-Acid Substitutions of MV for Adaptation to Vero Cells

Eight Vero-adapted strains belonging to the D5 and D9 genotypes were obtained after 4–22 passages: AN-V4, OY-V4, OY-V22, TY-V4, TY-V22, MA-V15 and KU-V4 from five wild-type D5 strains and D9-V4 after four passages of wild-type D9 strain. Original wild-type strains did not show any cell fusion in Vero cells and eight Vero-adapted strains were obtained, demonstrating syncytia formation. No mutation was noted in the F gene in the strains studied and amino-acid substitutions in the M and H proteins are shown in table 1. Regarding the H gene of the Vero-adapted strains, an amino-acid substitution at position 481 from Asn to Tyr (N481Y) was noted in OY-V4 and MA-V15. An amino-acid substitution at position 546 from Ser to Gly (S546G) was observed in AN-V4, TY-V22 and KU-V4. OY-V22 showed substitutions at positions 481 and 546. No mutation was observed for TY-V4 and D9-V4. With respect to the M gene, substitutions were observed at position 62 of the M protein from Ser to Arg (S62R) and at position 83 from Ser to Pro (S83P) of OY-V22, and at position 89 from Glu to Gly (E89G) of D9-V4. The mutation site(s) for adaptation to Vero cells was different from strain to strain.

For each Vero-adapted MV strain, H and M expression plasmids were constructed, and the results of sequence analyses are shown in table 2. Among ten H expression plasmids derived from the OY-V4 strain, four had N481Y and the remaining six were wild type. Among 12 H expression plasmids of OY-V22, three had N481Y substitution, six had S546G, one had both N481Y and S546G, and the remaining two were the original wild type. Six H expression plasmids of MA-V15 showed N481Y substitution. Six of seven plasmids derived from

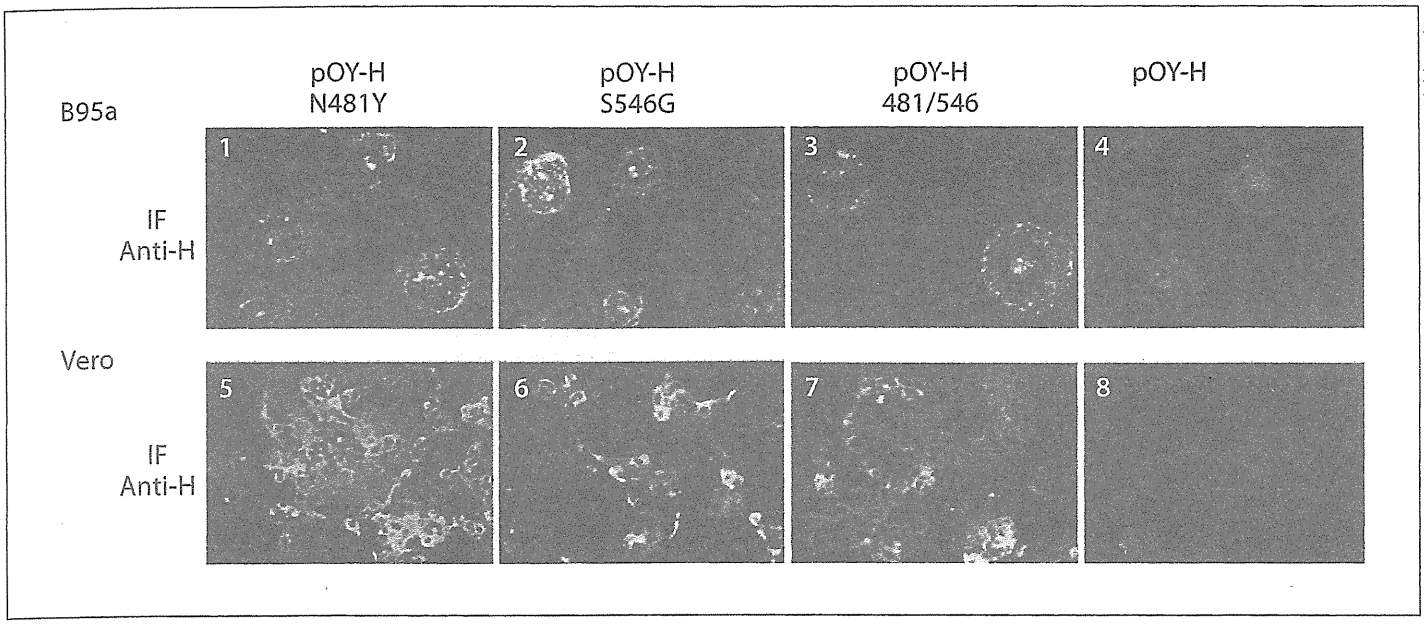

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Fig. 2. Fusion experiment involving H protein expression plasmids. The H protein expression plasmids were constructed: pOY-H was constructed from MVi/Mie.JPN/19.07, pOY-H N481Y, pOY-H S546G and pOY-H 481/546 from OY-V22 (Vero-adapted strain from MVi/Mie.JPN/19.07-OY). They were co-transfected with the F expression plasmid of the AIK-C in B95a cells (upper panels) and Vero cells (lower panels). The appearance of cell fusion and the results of IF using monoclonal antibody against H protein.

TY-V22 and all seven from KU-V4 showed S546G substitution. For the M gene, substitutions of S62R and S83P were observed in OY-V22 and that of E89G in D9-V4. Among 24 M gene clones derived from OY-V22, there were two clones with S62R and the remaining 22 had both S62R and S83P substitutions. As for the D9-V4 strain, four plasmids out of six M gene clones showed E89G substitution.

Expression Experiments Involving F and H Proteins

Through cloning experiments, the H protein expression plasmid was constructed from the original MVi/Mie.JPN/19.07-OY strain (pOY-H), and three expression plasmids with mutation(s) derived from OY-V22 (Vero-adapted strain of MVi/Mie.JPN/19.07-OY strain) were constructed: pOY-H N481Y (with an amino-acid substitution at position 481 of H protein), pOY-H S546G (with an amino-acid substitution at position 546) and pOY-H 481/546 (with two substitutions). pAIK-F was used as an F expression partner constructed from the AIK-C vaccine strain. The H expression plasmids were co-transfected with pAIK-F as the F expression partner in B95a or Vero cells, and the results of indirect IF staining are shown in figure 2. They induced a similar level of cell fusion in B95a cells, but the original wild-type pOY-H did not induce cell fusion in Vero cells (panel 8). The plasmids with an amino-acid substitution of N481Y (pOY-H N481Y) or S546G (pOY-H S546G) induced cell fusion in both B95a and Vero cells, and plasmid with both N481Y and S546G substitutions (pOY-H 481/546) induced more prominent cell fusion in Vero cells (panel 7).

Construction of Recombinant MV Strains with H Mutations

Recombinant cDNAs having wild-type original H gene of MVi/Mie.JPN/19.07-OY and mutated H gene from the Vero-adapted strains (OY-V22) were constructed based upon AIK-C cDNA, and infectious viruses were recovered: rMV-OY H without amino-acid substitution, rMV-OY H N481Y with amino-acid substitution of N481Y, rMV-OY H S546G with amino-acid substitution of S546G and rMV-OY H 481/546 with both substitutions. These recombinant MV strains were designed to express GFP, and the expression of GFP and IF staining against N protein are shown in figure 3. rMV-OY H did not show cell fusion in Vero cells similar to the original wild-type MVi/Mie.JPN/19.07-OY (panels 13, 18), but the Vero-adapted strain OY-V22 induced cell fusion in Vero as well as B95a cells (panels 5, 14). rMV-OY H N481Y and rMV-OY H S546G induced cell fusion in Vero cells to a

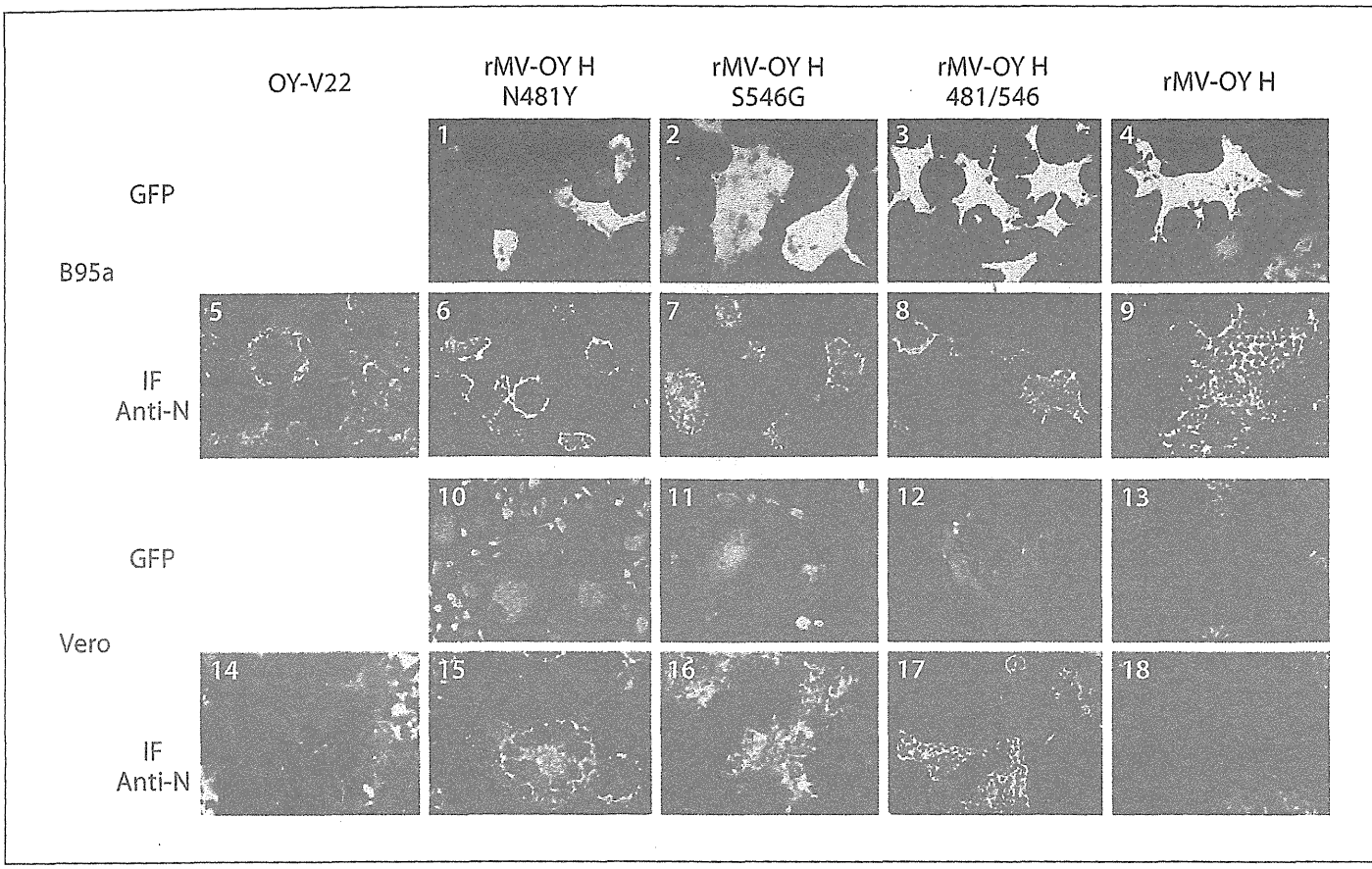

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Fig. 3. GFP expression and IF staining of B95a and Vero cells infected with recombinant MV strains with substitutions of H protein. OY-V22 was a Vero-adapted strain after 22 passages in Vero cells. Four recombinant MV strains were constructed: rMV-OY H had no mutation, and rMV-OY H N481Y, rMV-OY H S546G and rMV-OY H 481/546 had mutations at the respective positions. They were used to infect B95a and Vero cells, and the results of GFP expression and IF using a monoclonal antibody against measles N protein are shown.

similar extent, and rMV-OY H 481/546 led to slightly more extensive fusion in Vero cells.

Construction of Recombinant MV Strains with M Gene Mutations of OY-V22

Two amino-acid substitutions were noted in the M gene of the OY-V22 strains, S62R and S83P. The AIK-C vaccine strain has Tyr (Y) at position 481 of the H region, and mutation was introduced to generate Asn (N) at 481 (rMVAIK H481N). The M gene of the AIK-C was replaced by that of OY-V22 in the pMVAIK H481N cDNA, and two recombinant MV strains were recovered: rMVAIK H481N/M S62R and rMVAIK H481N/M 62/83. These M gene mutations were introduced into the cDNA of rMV-OY H and rMV-OY H/M S62R and rMV-OY H/M 62/83 were recovered. GFP expression and expression of measles N protein are shown in figure 4. rMVAIK H481N did not induce cell fusion in Vero cells (panels 11, 16), but rMVAIK H481N/M S62R and rMVAIK H481N/M 62/83 strains induced cell fusion (panels 12, 13, 17, 18). rMV-OY H/M S62R and rMV-OY H/M 62/83 strains induced cell fusion in Vero cells (panels 14, 15, 19, 20).

Virus Growth of Recombinant MV Strains with H and M Gene Mutations

All recombinant MV strains were designed to express GFP and virus growth was monitored by GFP expression, measured as fluorescence units (FU). The cell-free infectious virus titer was examined on days 1, 3, 5 and 7 after infection. The results of virus growth of recombinant MV strains with mutations in the H gene are shown in figure 5. Baseline GFP expression was <400 FU. rMV-OY H (Hwt) failed to produce syncytia in Vero cells without GFP expression, similar to the fusion experiment using

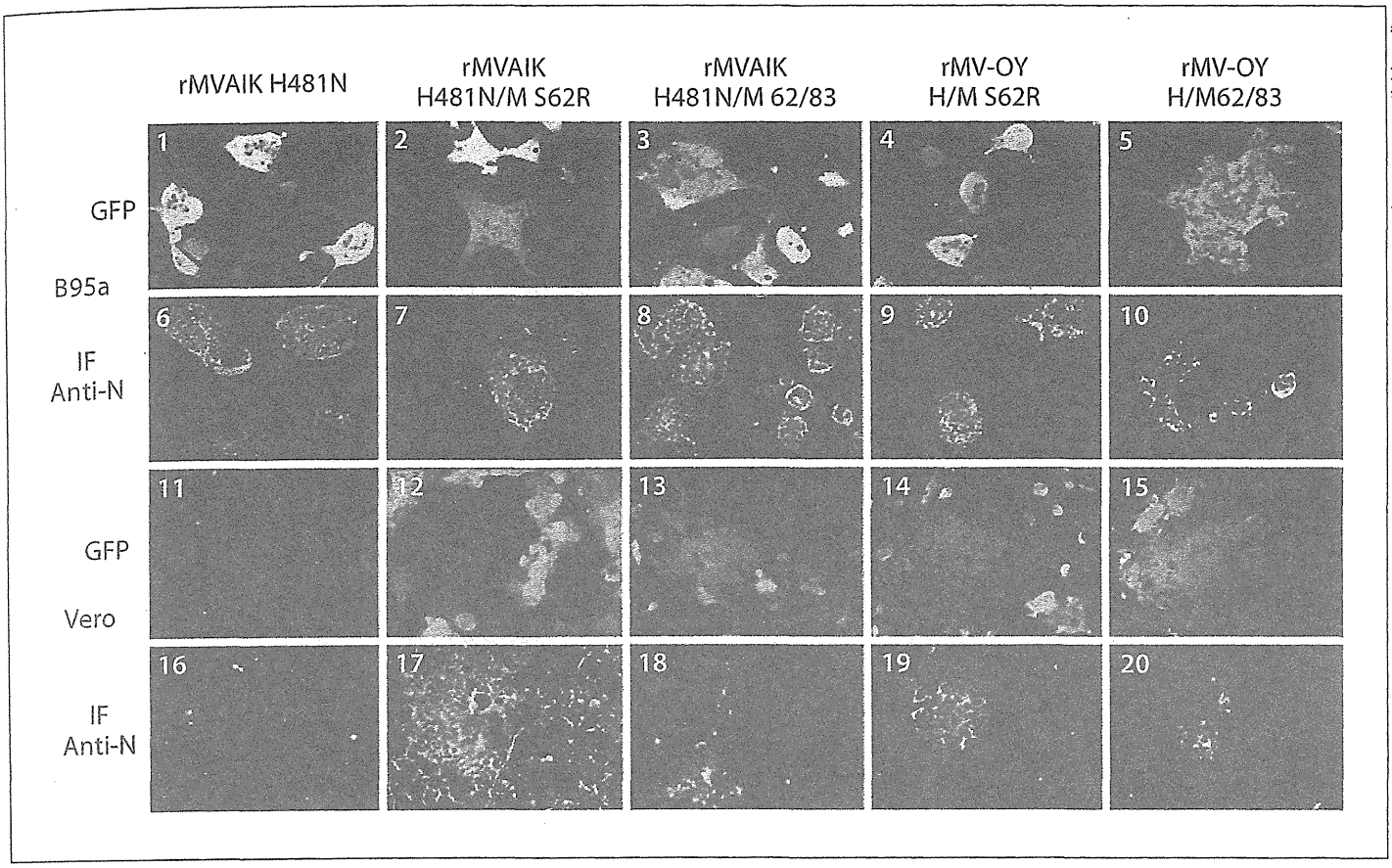

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Fig. 4. GFP expression and IF staining of B95a and Vero cells infected with recombinant MV strains with M protein substitutions. The M gene of AIK-C was replaced with that obtained from OY-V22 with mutation of S62R, S62R/S83P, and the H gene was also replaced by OY-H wild-type (rMV-OY H/M S62R, rMV-OY H/M 62/83). The M gene mutations were also introduced into rMVAIK H481N, rMVAIK H481N/M S62R and rMVAIK H481N/M 62/83. The results of GFP expression and IF using a monoclonal antibody against measles N protein are shown.

the H expression plasmids, and no infectious virus was observed in the supernatants. rMV-OY H 481/546 (H481/546) grew better on day 5 or 7 after infection with a high infectious titer of 10^5 TCD_{50} and high GFP expression over 2,500 FU in Vero cells, and induced more marked fusion in Vero cells than in the other recombinant MV strains with N481Y or S546G substitution. rMV-OY H S546G (H546) induced higher GFP expression than rMV-OY H N481Y (H481), but there were no significant differences in the production of infectious virus particles. In B95a cells, four recombinant MV strains demonstrated similar infectious virus production, but the rMV-OY H S546G strain led to a lower expression of GFP than rMV-OY H, rMV-OY H481N and rMV-OY H 481/546 (fig. 5).

GFP expression and the production of infectious virus in Vero and B95a cells infected with recombinant MV strains with M gene mutation(s) are shown in figure 6. M gene mutation(s) was introduced into rMVAIK-H481N or rMV OY-H. rMV OY-H (Hwt) did not express GFP and no infectious virus particle was produced in Vero cells. rMV-OY H/M S62R (M62/Hwt) and rMV-OY H/M 62/83 (M62/83/Hwt) induced GFP expression, and an infectious virus titer of $10^{2.0-3.0}$ TCD_{50} was obtained in the supernatants on day 7 of Vero cell culture. rMV-OY H induced extensive cell fusion in B95a cells with higher level of GFP expression and particle formation, but four other recombinant MV strains with M gene mutation(s) induced lower GFP expression with a lower production of infectious particles. Although recombinant MV stains with mutated M protein produced cell fusion in Vero cells, they induced lower cell fusion with lower numbers of fusion foci in comparison with those produced in B95a cells.

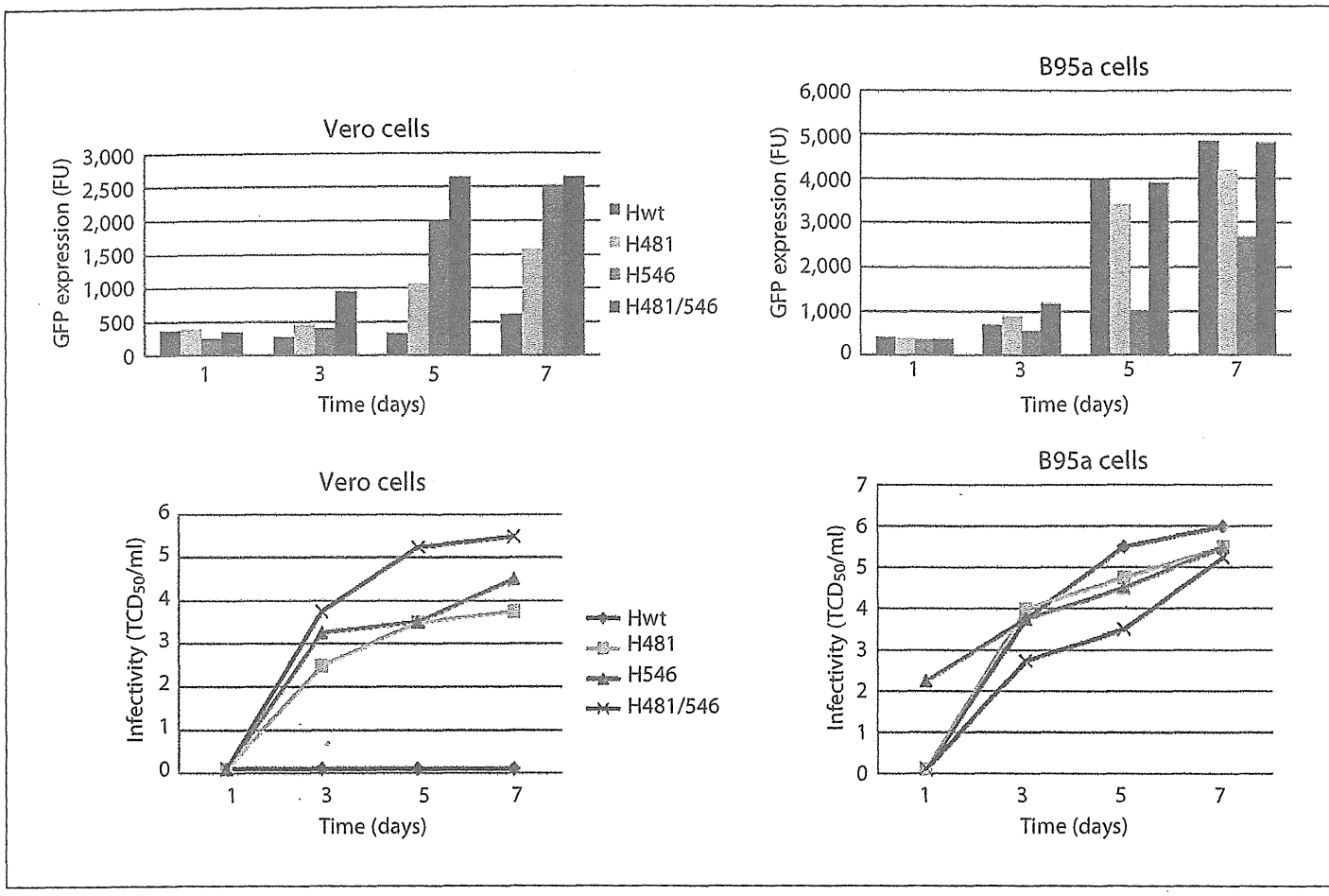

Fig. 5. GFP expression and virus growth of recombinant MV strains with substitution of H protein. Vero and B95a cells were infected with rMV-OY H (Hwt), pOY-H N481Y (H481), pOY-H S546G (H546) and pOY-H 481/546 (H481/546) strains at MOI = 0.01. GFP expression was monitored on days 1, 3, 5 and 7 after infection, and infectivity in culture supernatants was assayed (baseline GFP expression: 200–300 FU).

Recombinant MV Strains Derived from D9 Strain

D9-V4 was a mixture of M gene mutation. Four clones showed mutation at position 89 of the M gene from Glu to Gly, and the remaining two clones showed no mutation. The H and M genes of AIK-C cDNA were replaced with those amplified from the D9-V4 strain. rMV-D9 H/M has the original wild-type H and M genes, and rMV-D9 H/M E89G has wild-type H gene and E89G mutation of the M gene, similar to the D9-V4 strain. The results of cell fusion, GFP expression and infectivity in culture fluids are shown in figure 7. Two recombinant MV strains showed similar cell fusion in B95a cells, with similar infectious virus production. Whereas rMV-D9 H/M E89G induced cell fusion and produced infectious virus particles ($10^{3.1}$ TCD_{50}/ml) in Vero cells, rMV-D9 H/M did not induce cell fusion and showed no virus growth in Vero cells.

Discussion

MV induces extensive syncytium formation with cell fusion, and the appearance of a syncytium is a positive indicator of virus isolation. Binding of the H protein to the receptor induces the conformational changes of H and F proteins required for the protrusion of the fusion domain into lipid bilayers of the cell membrane [3–5]. At present, two MV receptors have been identified: CD46 and SLAM (CD150). CD46 is expressed widely on the surface of epithelial cells, including Vero cells, whereas CD150 is a lymphocyte-stimulating factor expressed on the surface of lymphoreticular cells [10, 31]. In the past, MV was isolated after three or more blind passages in Vero cells, and the isolation rate was low. Otherwise, current wild-type MV strains were isolated in B95a cells. The

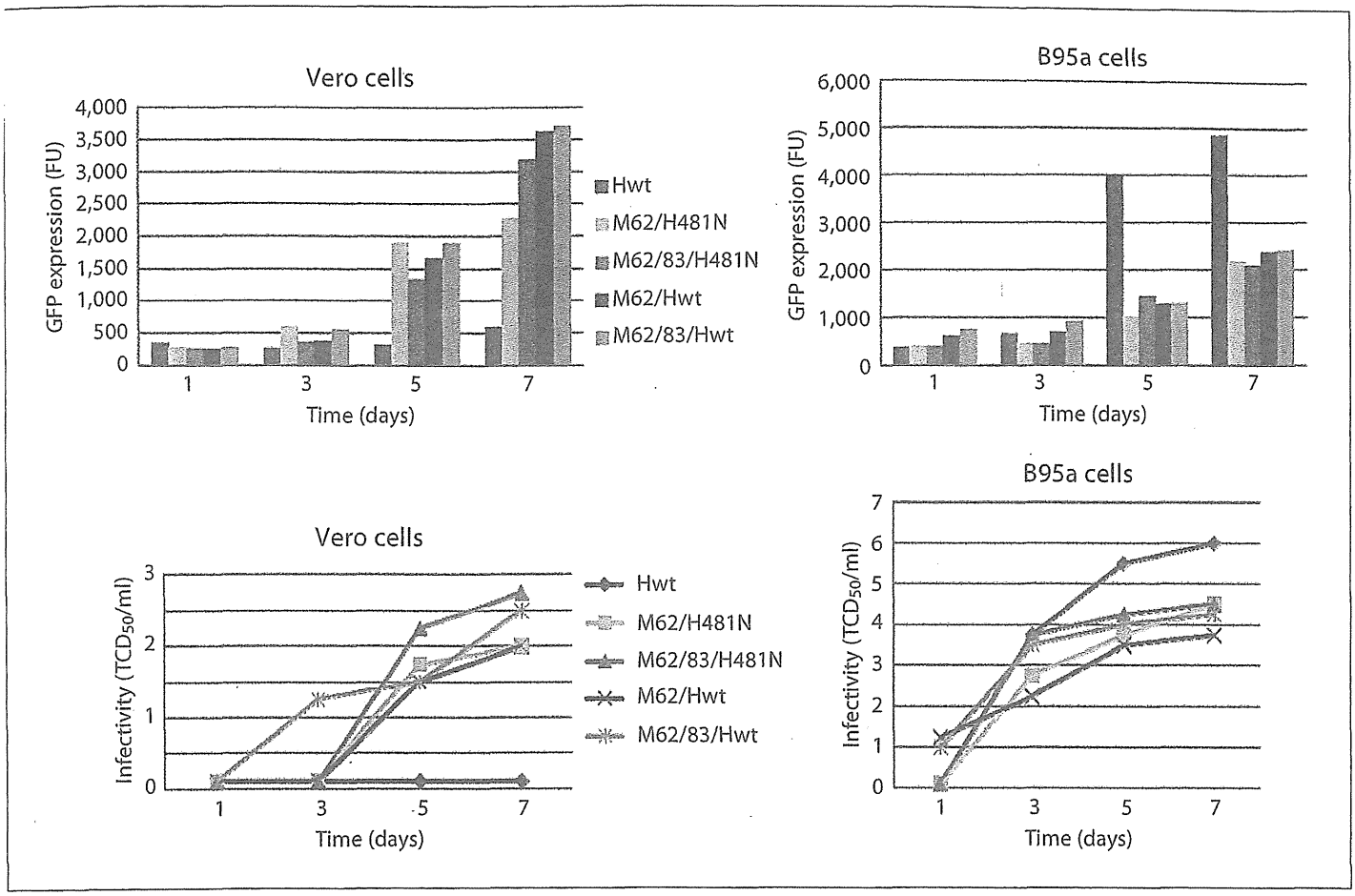

Fig. 6. GFP expression and virus growth of recombinant MV strains with substitution of M protein. Vero and B95a cells were infected with rMV-OY H (Hwt), rMVAIK H481N/M S62R (M62/H481), rMVAIK H481N/M 62/83 (M62/83/H481N), rMV-OY H/M S62R (M62/Hwt) and rMV-OY H/M 62/83 (M62/83/Hwt). GFP expression was monitored on days 1, 3, 5 and 7 after infection, and infectivity in culture supernatants was assayed (baseline GFP expression: 200–300 FU).

adaptation of the current wild MV to Vero cells led to amino-acid substitution(s) in the H gene that increased the binding capacity of the measles H protein to CD46 [19]. Lecouturier et al. [12] reported that substitutions of two amino-acid positions of 451 and 481 in H protein of the Halle strain abrogated the fusion inducibility of the functional domain(s) of the measles H protein. Hsu et al. [14] reported that a single amino-acid change at position 481 determined the ability of H protein to bind CD46. Xie et al. [15] reported that Asn at position H481 of the wild-type expression plasmid was replaced by various amino acids, and the mutant plasmid with Tyr, similar to the Edmonston strain, induced cell fusion, but this substitution did not cause the down-regulation of CD46 expression, unlike the Edmonston strain. Thus, Tyr at position 481 was indispensable for measles H protein to interact with CD46, similar to the other reports [16, 21]. A single substitution of N481Y of the wild H protein was not sufficient to use CD46, suggesting that further substitutions were required for efficient virus growth in Vero cells [22]. From the comparison of the H gene of the Edmonston and current circulating strains, three substitutions (N309I and E492G, plus N416D or T446S) were necessary for efficient virus growth in Vero cells [32]. Li and Qi [20] examined the amino-acid substitutions of MV H protein when three hemadsorption-negative strains were passaged >20 times in Vero cells. They reported that amino-acid substitution at position 546 of the measles H protein from Ser to Gly was critical for hemadsorption and CD46 binding besides the amino-acid change at position 481. In the three-dimensional surface representation of the structural model, three of these residues (D505, D507 and

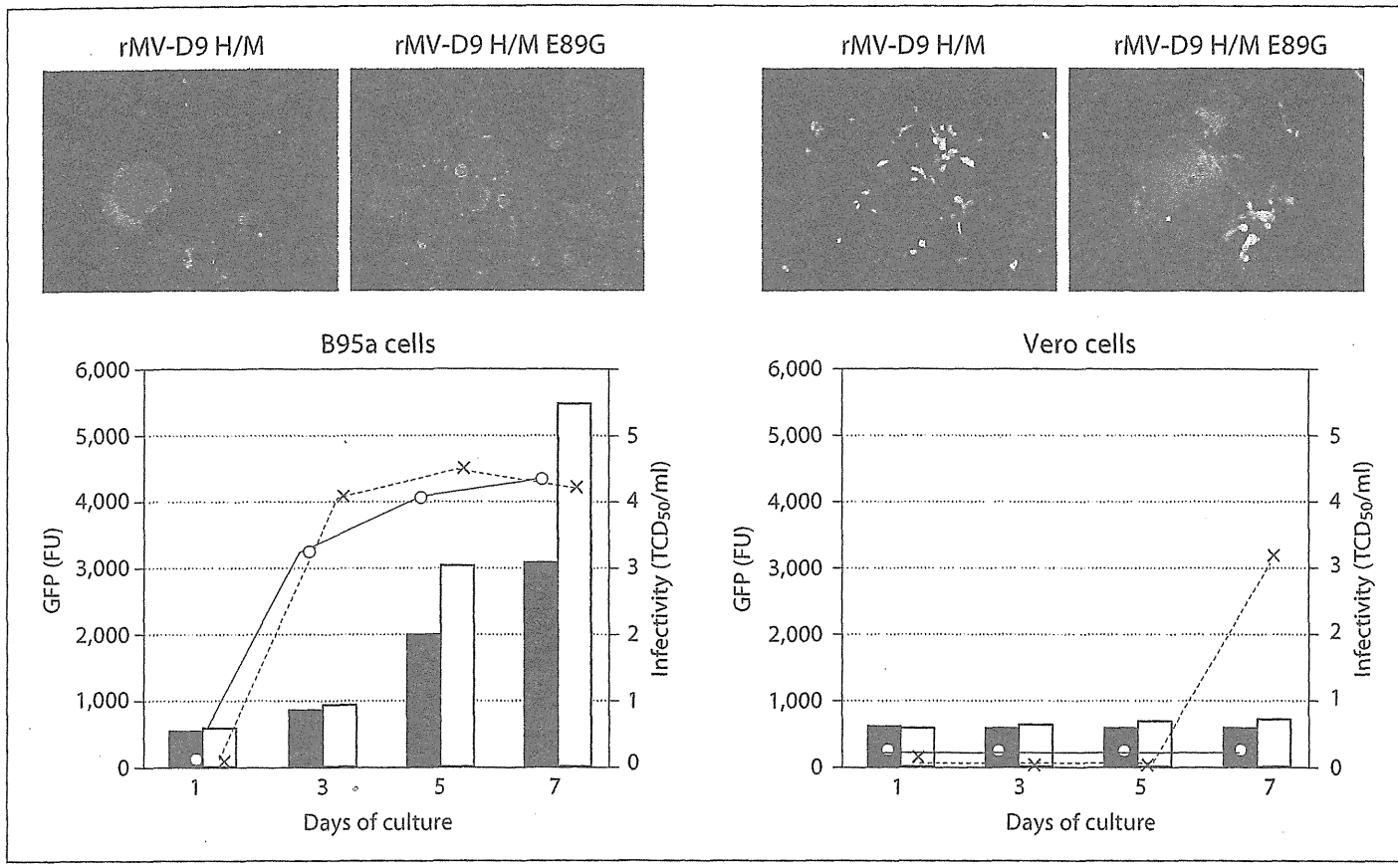

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Fig. 7. Characteristics of recombinant MV strains with a mutation in M protein observed in the D9-V4 Vero-adapted strain. The M and H genes of AIK-C were replaced with those of MV/Aichi.JPN/44.06 (rMV-D9 H/M), and the mutated M gene was introduced (rMV-D9 H/M E89G). GFP expression is shown in the upper panels. Vero and B95a cells were infected, and GFP expression (■; □) and virus infectivity (——; - - -) were monitored. ——○——/■ = rMV-D9 H/M; - - × - -/□ = rMV-D9 H/M E89G.

R533) align along the rim on one side of the cavity on the top surface of the measles H globular head, and form the basis of a single continuous site that overlaps with the 546-548-549 CD46 binding site. Mutations at position 481 or 546 induce conformational changes in the measles hemagglutinin globular head and influence the affinity for CD46 binding [33]. The MV H protein three-dimensional approach suggested that the SLAM- and CD46-relevant residues are located in contiguous areas in propeller β sheets 5 and 4, respectively, and several CD46-relevant amino acids may be shielded from direct receptor contact [34–36]. Using the Edmonston H protein, the association rate for SLAM binding to H protein was very low; about 20 times lower than CD46. However, SLAM bound to H protein more tightly than CD46, as revealed by a 5-fold lower dissociation rate [37].

The Vero-adapted MV strains showed several amino-acid changes in the other genes: two in the P, V and C proteins, three in the H gene (Ala H14 Gly, Leu H423 Pro and Ser H546 Gly) and two in the L gene [13]. Only two nucleotide differences were reported at 2331 genome position of P/V/C and at the 3628 genome position (amino acid position 64 of M protein), and none in the H gene between the MV genome isolated in B95a cells and that isolated in Vero cells from the same patient [38]. Through comparative studies of the M gene sequence of wild-type and Edmonston strains, substitutions of P64S and E89K from wild-type M protein were reported to be responsible for the fusion inducibility and efficient virus growth in Vero cells [23]. These two substitutions (P64S and E89K) allowed the interaction of the M protein with the cytoplasmic tail of H protein, thereby enhancing cell fusion and the assembly of infectious particles in Vero cells [24]. M protein also had binding activity to the cytoplasmic domain of H protein together with F protein and ribonucleoprotein complex, and was transported to the mem-

brane raft fraction [25, 39, 40]. In this report, no substitution was noted in the F protein for adaptation to Vero cells but substitutions were reported at positions 439 and 464 of the F protein, as well as those in the N, P/V/C, H and L proteins after adaptation to Vero cells [41].

In this report, cell fusion was observed after four passages of MVi/Mie.JPN/3.07-TY/B3 (TY-4 strain) in Vero cells but TY-4 had no amino-acid substitution in the M, F and H proteins. There was a possibility of mutation(s) in the P and/or L genes and, after 22 passages, substitution of H546G was detected. Amino-acid substitutions for adaptation to Vero cells were different from strain to strain. All D5 genotype strains showed a substitution at position 481 or 546 of the H protein region at an early stage of passage. These were mutated through the adaptation process, and no substitution except for those at these positions was observed on direct sequencing analysis. Thus, the substitutions of N481Y or S546G were essential for adaptation to Vero cells, and the other regions were not changeable. OY-V4 showed a mixed population of N481Y and original wild-type clones, whereas OY-V22 was a mixture of the N481Y substitution, S546G, substitutions of both N481Y and S546G, and the original sequence. Through the results of expression experiments involving H plasmids and GFP expression of recombinant MV strains, rMV-OY H 481/546 induced more extensive cell fusion in Vero cells than MV strains with either of the substitutions. Double mutants were predicted to use CD46 more efficiently, leading to efficient infectious virus production and growth in Vero cells.

Further repeated passages in Vero cells accumulated mutations in the M gene in addition to the H gene. Two strains adapted to Vero cells showed substitutions in M protein. Substitution of E89G was observed in D9-V4, and those of S62R and S83P in OY-V22. rMV-OY H/M S62R and rMV-OY H/M 62/83 strains induced cell fusion in Vero cells. These recombinant MV strains decreased virus growth and particle formation in B95a cells and the single substitution of S62R was sufficient. These positions were different from those in previous reports, demonstrating the fusogenicity of combined substitutions of P64S and E89K in Vero cells [23, 24, 32]. The backbone of our reverse genetics is the AIK-C vaccine strain and rMV H481N and rMV-OY H have K at position 89 of M protein, but these two strains did not induce cell fusion without infectious virus production. Thus, the single mutation of E89K would not be a critical region for interaction between M and H proteins for efficient virus growth in Vero cells. Substitution of E89G of the M protein was observed in D9-V4. rMV-D9 H/M (E at position 89 of M protein) did not induce cell fusion, with no virus growth, but GFP was demonstrated in cells without fusion. rMV-D9 H/M E89G induced a small fusion with a low level of infectious virus production, even though it had wild-type H protein. This may suggest the presence of another unidentified receptor for MV and, recently, the possibility of a molecule related to tight junctions on the basolateral sides of epithelial cells was reported [42, 43].

Adaptation would occur in a different manner depending on the strains or experimental condition. Three strains showed mutations in the H gene within four passages, and repeated passages added additional mutations to the M and H genes. One strain of D9 showed a substitution in the M protein, even without H protein substitution at N481Y or S546G which enables the virus to enter cells efficiently. Thus, we supposed that the amino acids at positions 481 and 546 of H protein are critical for the different tropisms based on the results of expression experiments. From the results of recombinant MV strains with M protein mutations, substitution in the M protein promoted efficient MV growth and particle formation in Vero cells, and would influence efficient receptor usage of the wild-type H protein to induce cell fusion irrespective of H gene mutation.

References

1 Morrison TG: Structure and function of a paramyxovirus fusion protein. Biochim Biophys Acta 2003;1614:73–84.
2 Griffin DE: Measles virus; in Knipe DM, Howley PM (eds): Fields Virology, ed 5. Philadelphia, Lippincott-Raven/Williams and Wilkins, 2007, pp 1551–1585.
3 Wild TF, Malvoisin E, Buckland R: Measles virus: both the haemagglutinin and fusion glycoproteins are required for fusion. J Gen Virol 1991;72:439–442.
4 Lamb RA: Paramyxovirus fusion: a hypothesis for changes. Virology 1993;197:1–11.
5 Lamb RA, Paterson RG, Jardetzky TS: Paramyxovirus membrane fusion: lessons from F and HN atomic structures. Virology 2006; 344:30–37.
6 Kobune F, Sakata H, Sugiura A: Marmoset lymphoblastoid cells as a sensitive host for isolation of measles virus. J Virol 1990;64: 700–705.
7 Dörig RE, Marcil A, Chopra A, Richardson CD: The human CD46 molecule is a receptor for measles virus (Edmonston strain). Cell 1993;75:295–305.
8 Naniche D, Varior-Krishnan G, Cervoni F, Wild TF, Rossi B, Rabourdin-Combe C, Gerlier D: Human membrane cofactor protein (CD46) acts as a cellular receptor for measles virus. J Virol 1993;67:6025–6032.
9 Tatsuo H, Ono N, Tanaka K, Yanagi Y: SLAM (CDw150) is a cellular receptor for measles virus. Nature 2000;406:893–897.