Molecular epidemiology of Nigerian and Ghanaian measles virus isolates reveals a genotype circulating widely in western and central Africa

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Sub-Saharan Africa is one of the regions of the globe with the highest measles-related morbidity and mortality. Yet only seven virus isolates from this vast region have been phylogenetically characterized on the basis of their nucleoprotein, the last one in 1991. To characterize the prevalent wild-type viruses and to understand their circulation pattern, a large panel (n = 45) of isolates was collected in Ghana and Nigeria in 1997 and 1998. On the basis of their nucleoprotein sequence, the viruses clearly belong to clade B but a reshuffling of the structure of this clade was proposed, tentatively extending the number of genotypes from two to three on the basis of quantitative criteria. The sequences revealed the co-circulation of at least two distinct viruses in the cities of Lagos and Ibadan, suggesting that the number of susceptible individuals seems to be high enough to support endemic circulation of at least two distinct viruses. The endemic co-circulation of several viruses may well be a characteristic of communities with low vaccination rates. One of these viruses was also found in Accra in 1998 as well as in a 1994 case linked to distant Kenya, suggesting that clade B viruses are prevalent in sub-Saharan Africa while non-B viruses seem to dominate the south of Africa.

Introduction

Measles virus (MV) is the prototype and the only known human pathogen of the morbilliviruses. Since it is considered to be monotypic and essentially without animal reservoir (Norrby, 1995), measles control and eradication should be within reach of aggressive vaccination campaigns. To achieve this goal, it is essential that national health efforts concentrate on strategies to interrupt virus circulation. Molecular epidemiology of field isolates has proven to be a powerful tool to monitor circulation of infectious virus (Rota et al., 1994b, 1996; Rima et al., 1995; Kreis et al., 1997). In the Americas, the molecular analysis of virus isolates has demonstrated that virus circulation has been effectively disrupted and has ceased in large parts of the continent (Rota et al., 1996; Bellini & Rota, 1998). However, in most other continents, the virus is still endemic (WHO, 1997) and virus transmission continues within geographically more or less confined areas without the need for reintroduced exogenous virus. In some regions, specific virus genotypes have established themselves. A comprehensive study of isolates from different geographic regions will permit the discrimination between imported and endogenous cases. In countries where measles seems to be under control, the geographic origin of reintroduced virus can potentially be determined. Genotype variability and distribution can help to assess the impact of vaccination efforts in endemic areas.

No field isolates from the vast region of West Africa, including Nigeria, the most populous African nation, have ever been studied. Except for some rare isolates from Cameroon and Gabon from the mid-1980s and Gambian isolates in the 1990s,
Table 1. Isolates collected from measles patients

Hospitals involved were Adeoyo State Hospital (ASH), Oni Memorial Children Hospital (OMCH), University College Hospital (UCH), St Mary’s Hospital (SMH), Massy Children’s Hospital (MCH), Lagos University Teaching Hospital (LUTH), Infectious Disease Hospital (IDH), Princess Mary Children’s Hospital (PMCH). Accession numbers are from the EMBL database.

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no MV field strains have ever been investigated in northern and central Africa (Taylor et al., 1991; Rota et al., 1994b; Outlaw et al., 1997). Yet, this may well be the global region with the highest measles morbidity and mortality (Dollimore et al., 1997; WHO, 1996). In Nigeria, for instance, measles is endemic and the death toll is exceedingly high in young infants (Byass et al., 1995; O. Ikusika & F. Adu, unpublished data). We describe here the sequence variability of the nucleoprotein (NP) gene of a large panel of MV isolated recently in Ibadan and Lagos and neighbouring Ghana. On the basis of the variable C-
terminal domain of the NP (Taylor et al., 1991; Rima et al.,
1995), the isolates were tentatively assigned to a new genotype of
clad B. Sequence comparison with earlier isolates con-
tributes to a better understanding of measles epidemiology in
sub-Saharan Africa.

Methods

■ Patients. Most viruses were isolated in February and March 1998
from blood or throat-swabs of patients (7–108 months of age) with
clinical measles. Three additional isolates were obtained in April
and November 1997 (Table 1). Samples were taken within 6 days after onset
of the rash. Four samples (Ibadan.NIE/7.98/2, Ibadan.NIE/9.98/8,
Ibadan.NIE/10.98/6, Accra.GHA/9.98/1) were drawn from children of
6–9 months of age, i.e. before the recommended age of vaccination of 9
months. Sixteen children were vaccinated according to their parents’
memory and 21, including the above four children below 9 months of
age, were reportedly not vaccinated. The vaccination status of 10
patients was unknown. The Ibadan samples were collected mainly from
the paediatric outpatient departments of three public hospitals [Adeoyo
State Hospital (n = 15), Oni Memorial Children Hospital (n = 13),
University College Hospital (n = 4) and one private hospital (St Mary’s
Hospital; n = 3). Samples from Lagos were collected in the Massey
Children’s Hospital (n = 2), the Lagos University Teaching Hospital (n
= 2) and the Infectious Disease Hospital (n = 1). All samples from
Ghana were from the Princess Mary Children’s Hospital, Accra (n = 4).
Peripheral blood lymphocytes or the cellular fraction of throat-swabs
were co-cultivated with a semi-confluent monolayer of B95a cells (an
Epstein–Barr virus-transformed marmoset B cell line of lymphoblastoid
origin; Kobune et al., 1990). Cytopathic effect appeared after 1–14 days.
The complete culture was harvested, aliquoted and stored in liquid
nitrogen or at −20 °C.

■ Sequencing. Total RNA was extracted using a modified
guanidinium–acid phenol method (Chomczynski & Sacchi, 1987). In brief,
cells were incubated with Trizol (GIBCO Life Technologies) and
RNA was extracted with chloroform and precipitated in isopropanol.
Specific cDNA of MV NP mRNA was synthesized by reverse
transcription using Superscript Reverse Transcriptase (GIBCO Life
Technologies) and primer NP4rev (ACAATGATGGAGGGTAGGCG-
AGGGATG). The cDNA was further amplified using the primers NP1seq
(TTCAAGATCTTATTCAGGGACAAG) and NP4rev to obtain
a 1676 bp fragment, and by semi-nested PCR with NP1seq/NP2rev
(TTCAAGATCTTATTCAGGGACAAG) and NP3seq (TTGCTGGTG-
AGTTATCCACACTTG)/NP4rev, respectively, to increase the speci-
ficity. The resulting two fragments, each corresponding to about one-half
of the gene (985 and 763 bp long) were purified in a 1% agarose gel.
RT–PCR products were directly sequenced with the Dye Terminator
Cycle Sequencing kit (Perkin-Elmer) and four specific primers for each
strand were used in addition to the above primers, NP1rev (GGCTCA-
AGGATGATGACCAGAATC), NP2seq (TTTCTAGATGTGATCAGCCA-
ATTAGTATG), NP3rev (GACTCCGATGCTGATCAGCT) and
NP4seq (GAGAAGCGGGCCACACAGA). Sequencing was per-
formed on an ABI 377 Automatic DNA Sequencer (Perkin-Elmer).
Sequence data were analysed using the CLUSTAL X program (Thompson
et al., 1994) for multiple alignments and a version of the PHYLIP package
(Felsenstein, 1993) running on the HUSAR computer at the DKFZ
(Heidelberg, Germany) for phylogenetic analysis. Dendrograms
were drawn using the neighbour-joining method and confirmed with 1000
bootstrap replicates. Similar results were obtained with the maximum
likelihood method and 100 bootstrap replicates (data not shown).
All virus isolates, genotypes and clades were designated according to the
new official WHO nomenclature (WHO, 1998). Sequences used for
comparison in Fig. 3 were described by Taylor et al. (1991) (Y22, R118,
JM, MVO and MVP), Rota et al. (1994a) (Edm. wt, Edm. B, CAM 70 and
Shanghai), Rota et al. (1994b) (Y14, R103, R113, Chicago 1, CO 94,
Gum 94, Canada 89, Berkeley 83 and Boston 83), Rima et al. (1995) (MF,
Mad 79, WTF, Brx, WFK, SMA 79 and SMA 94), Rota et al. (1996)
(Gambia 91, Palau 94, NJ 94 and ENG 93), Kreis et al. (1997) (Jhb 1/86,
Jhb 1/88 and Jhb 3/94) and Xu et al. (1998) (China 1/93 and China 7/93).

Results

Sequence variability among the isolates
Since the virus isolates described here are the first from this
major region of West Africa (Nigeria and Ghana) the complete
protein-encoding region of the NP gene was sequenced. The
nucleotide divergence in the NP gene among our isolates
varied from 0·0 to 2·6% but only about one-third translated
into mutated amino acids.

Comparison with a consensus sequence
The sequences of four isolates from Accra (Ghana), five
from Lagos and 36 from Ibadan (both Nigeria) were compared to
a consensus sequence based on 34 selected MV NP
sequences of the EMBL database covering all genetic sub-
grroups defined by the WHO (1998). The 456 nucleotides
at the 3′-terminus were identical with the consensus sequence
described by Rima et al. (1995) and by Taylor et al. (1991). The
number of mutations in our isolates was in the range 35–44 at
the nucleotide level and 4–7 at the amino acid level. Particularly
in the C-terminal domain (nt 1230–1285), up to 3·7% of the
nucleotides displayed mutations, leading to a maximum of
seven amino acid substitutions. Within the N-terminal domain
(nt 108–1229), a maximum of 2·5% of the nucleotide positions
showed mutations, but almost all of these were silent at the
amino acid level. Within this domain, other variable regions
with a density of nucleotide mutations similar to the C-
terminal variable region seem to be embedded. In contrast to
the C-terminal terminations, these are mostly silent (data not
shown).

To identify the clade and the genotype to which our viruses
are most closely related, several types of comparisons were
made. (I) Fig. 1(a) shows that the divergence of four
representative Nigerian/Ghanaian isolates is lowest in compari-
son to the reference sequences of the genotypes of clades A
and B. (ii) Fig. 1(b) describes the number of shared mutations
between the reference sequences and the above representative
isolates with respect to the consensus sequence. The number of
mutations shared with clad B viruses is 2–9 times higher than
with reference viruses of other clades. (iii) In Fig. 2, the same
representative sequences are aligned with the clad-specific
consensus sequences and the archetypes for the B1 and B2
genotypes. The specific mutations of clad B are found in our
isolates. Mutation patterns are similar but not identical to the
B1 and B2 viruses. (iv) Finally, a dendrogram shows some
representative isolates of Table 1 together with the above
Fig. 1. (a) Nucleotide divergence (%) of four representative isolates, MV/ibadan.NIE/9.98/10 (dark grey bars), MV/ibadan.NIE/7.98/3 (black bars), MV/ibadan.NIE/8.98/9 (white bars) and MV/Accra.GHA/9.98/3 (light grey bars) in comparison with the archetype of each genotype. (b) The absolute number of nucleotide mutations that the isolates in (a) have in common with the genotype archetypes in comparison to the overall consensus sequence.

Fig. 2. Sequence alignment of the overall consensus sequence, the clade-specific consensus sequence showing the clade-characterizing mutations, the B1 and B2 archetypes and the four representative isolates shown in Fig. 1. The C-terminal variable region was truncated to 1254–1685 as the first 24 nucleotides did not show a mutation. Non-conserved amino acid positions are underlined.

Comparison with clade B isolates

All isolates in Table 1 share the following nucleotide substitutions: 383C → T, 470C → T, 473C → T, 494A → G, 542A → G, 548C → T, 623C → T, 668G → A, 701G → A, 719G → A, 767G → A, 1163T → C, 1214G → A, 1334G → A, 1394A → G, 1418T → C, 1449A → G, 1549C → T, 1583T → C, 1595C → T and 1673T → C (nucleotide numbering according to Mori et al., 1993). Thirteen of these mutations (in bold) are not found in the B2 subgroup (isolates R103, R113 and R118; Rota et al., 1994b; Taylor et al., 1991); four mutations (underlined) are not found in the B1 subgroup (Y22 and Y14; Rota et al., 1994b; Taylor et al., 1991). The sequences form two disparate clusters. Cluster 1 (n = 27) contains the 1997 isolates from Ibadan and 21 1998 isolates from Ibadan and three viruses from Lagos. Cluster 2 (n = 17) reference sequences (Fig. 3). Bootstrap values were significant for all clades but not for all genotypes as has been discussed previously (Rima et al., 1997). The above comparisons demonstrate that our isolates are most closely related to clade B.
comprises all isolates from Ghana, two viruses from Lagos and 11 viruses from Ibadan (all 1998). Within cluster 1, sequence divergence was maximally 0.8% (2.0% in the C terminus) and all sequences shared additional changes at positions 428T → C, 461A → G, 593A → C, 659C → T, 698A → C, 983G → A, 1079C → T, 1184A → G, 1436T → C, 1458T → C, and 1586C → T. In cluster 2, sequences varied by up to 1.7% (3.1% in the C terminus) with the common mutations 269G → A, 410G → A, 518T → C, 533C → T, 1127A → G, 1364T → C, 1434A → G and 1688G → A.

Only Lagos.NIE/10.98 lacked the changes at positions 269, 410, 1127, 1434 and 1688. All of the above-mentioned characteristic mutations were silent, except for the changes at 1434 leading to 443S → G, 1449 leading to 448R → G, 1458 leading to 451Y → H and 1549 leading to 481S → F (all located in the variable 456 nt of the C terminus).
A difference of 1.7–2.7% was found (1.8–4.6% in the C terminus) between the two clusters. The following differences were found with respect to earlier African isolates (Cameroon 1983 and Gabon 1984, B1 and B2, respectively, according to WHO nomenclature): between cluster 1 and B1, 2.4% (3.3% in the C terminus); cluster 2 and B1, 2.4% (2.6% in the C terminus); cluster 1 and B2, 3.2% (4.1% in the C terminus); cluster 2 and B2, 3.3% (4.4% in the C terminus) – an indication of a certain distance between the old and the recent isolates.

Discussion

Most of the isolates described in this study were collected in four hospitals within a two month period during a single outbreak in the city of Ibadan. Despite this narrow time-span and the limited out-reach, these isolates form a relatively heterogeneous group with up to 4.6% sequence divergence in the C-terminal variable domain of the NP. They represent two independent clusters with 11 and 8 specific mutations in clusters 1 and 2, respectively. Viruses of the two clusters were not confined to individual hospitals and were obtained throughout the outbreak. Local epidemics are mostly sustained by genetically homogeneous viruses. However, a similar heterogeneity was also observed by Xu et al. (1998) during an outbreak in China. During a long-term study of cases in Europe, a clade switch was observed (Rima et al., 1997) and, in the US, the upcoming of different genotypes was explained by genetically diverse, imported cases (Rota et al., 1996). Viruses of the two clusters also co-circulated in Lagos. Moreover, cluster 2 viruses were found at the same time in Accra, some 500 km away from Ibadan and cluster 1 viruses were already circulating in Ibadan in 1997. These observations suggest that the viruses were not introduced by a particular reference case but rather that they circulate endemically over a vast region where the number of susceptible individuals not only seems to be high enough to support the extensive circulation of a single virus but at least two distinct viruses. The endemic co-circulation of several viruses may well be characteristic for communities with low vaccination rates, if only sufficient numbers of viruses are sampled.

The new WHO nomenclature differentiates between eight distinct clades (named A–H), some of which may be extinct. Clades B, C and D comprise several genotypes. The dendrogram with our new isolates shows no major reshuffling in comparison to earlier versions, so the relationship between the groups should now be stable. Some more recent African isolates from South Africa (Kreis et al., 1997) and one from Kenya (Bellini & Rota, 1998) belong to clades that were mainly associated with Europe and the US. Our isolates clearly belong to clade B, which has always been associated with the African continent and which contains most of the earlier African isolates. However, our isolates are quite distinct from the prototypes of the subgroups B1 (isolated in Cameroon in 1983) and B2 (isolated in Gabon in 1984), in terms of the degree of divergent nucleotides, common mutations (in the 3’ variable region of NP) and bootstrap values. Among all known isolates, only NY 94 (Rota et al., 1996) shares all mutations common among the isolates of Table 1 with the exception of 767G → A. This 1994 isolate from New York corresponds to our cluster 2 with only 1% nucleotide sequence difference with its closest relatives (Ibadan.NIE/8.98/4, Ibadan.NIE/8.98/9, Ibadan.NIE/8.98/10, Ibadan.NIE/9.98/1). Interestingly, this virus was epidemiologically linked to an imported case from Kenya. This suggests that this highly stable virus may also circulate between Kenya and Nigeria, throughout sub-Saharan Africa. This is in line with our observation that cluster 2 viruses were also isolated in Ghana.

Although all our isolates clearly belong to clade B, it is difficult to assign them to one of the existing genotypes. The 45 isolates described here are bound to cause considerable reshuffling within the B clade from which, so far, the NP sequences of only seven viruses have been described. On the basis of the dendrogram and the mutations shared by our isolates but not shared by the clade B archetype sequences, we have provisionally assigned a new clade B genotype to our isolates from Nigeria and Ghana (tentatively B3), despite a low bootstrap value (43%). Although clade-specific bootstrap values are significant (> 80%), some well-established genotypes, such as D1, D2 and D5 also have low bootstrap values (50–67%). The reference virus of this genotype would be NY 94 since it was isolated first.

Due to its dissimilarity with both the B1 archetype and our viruses, the Gambia 91 isolate cannot be assigned to a genotype as long as no other comparable isolates are found. As shown below, the designation of a new genotype based on a single isolate is difficult since no shared mutations can be taken into account.

So far no criteria have been established to define the level of differences between clades and genotypes. The usefulness of a definition based on percentage difference, as proposed by Kreis et al. (1997) is limited, since it does not necessarily reflect a common evolution of viruses. A definition based on common characteristic mutations could be more useful but requires a larger number of virus isolates. It is proposed that a new clade is assigned to isolates which do not share most (65–80%) of the mutations found in a clade-specific consensus sequence as shown in Fig. 2. For clades with only limited numbers of known isolates (such as clades E and F, which seem to be extinct; WHO, 1998), the number of characteristic mutations may be artificially high. Within a clade, genotypes would be defined by the number of shared mutations that are only found in a given set of isolates. Two sets of viruses would belong to different genotypes when each group contains a minimal number of set-specific mutations. For instance, new isolates would be assigned to distinct genotypes if the sum of set-specific mutations of both sets adds up to at least five (on the C terminus). Within clade D, genotype 1 and 2 diverge by six characteristic mutations, D3 and D5 by 13 mutations, D3 and...
D1 by 15 mutations. According to the new clade B structure proposed above, the sums of the genotype-specific mutations are as follows: B1/B3, five mutations; B1/B2, 14 mutations; B2/B3, 13 mutations. It is important to note that the significance of these numbers increases considerably with the size of the compared sets of isolates, highlighting the need for collecting larger numbers of virus isolates.

The virus isolates described here are the cause of the staggering mortality rates among Nigerian infants and children. Although information concerning the vaccination status of the measles patients may not be very reliable in the absence of documented proof, it is likely that a considerable number of cases were due to primary and/or secondary vaccine failure. Vaccine handling as well as the poor nutritional and health status of children could explain low seroconversion rates.

This is the first phylogenetic study based on NP of a large number of MV isolates conducted in Africa with the exception of South Africa. Results of this study suggest that clade B viruses are prevalent in sub-Saharan Africa while non-B viruses seem to dominate the south of Africa (Kreis et al., 1998). Considering the size of the continent and the reservoir of susceptible individuals, it seems likely that other genotypes will emerge (Bellini & Rota, 1998) and that the diversity of known circulating MV will continue to grow.

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