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Spontaneous Mutation Rate of Measles Virus: Direct Estimation Based on Mutations Conferring Monoclonal Antibody Resistance

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High mutation rates typical of RNA viruses often generate a unique viral population structure consisting of a large number of genetic microvariants. In the case of viral pathogens, this can result in rapid evolution of antiviral resistance or vaccine-escape mutants. We determined a direct estimate of the mutation rate of measles virus, the next likely target for global elimination following poliovirus. In a laboratory tissue culture system, we used the fluctuation test method of estimating mutation rate, which involves screening a large number of independent populations initiated by a small number of viruses each for the presence or absence of a particular single point mutation. The mutation we focused on, which can be screened for phenotypically, confers resistance to a monoclonal antibody (MAb 80-III-B2). The entire H gene of a subset of mutants was sequenced to verify that the resistance phenotype was associated with single point mutations. The epitope conferring MAb resistance was further characterized by Western blot analysis. Based on this approach, measles virus was estimated to have a mutation rate of 9×10^{-5} per base per replication and a genomic mutation rate of 1.43 per replication. The mutation rates we estimated for measles virus are comparable to recent in vitro estimates for both poliovirus and vesicular stomatitis virus. In the field, however, measles virus shows marked genetic stability. We briefly discuss the evolutionary implications of these results.

The unique population structure and evolutionary dynamics of RNA viruses result in part from mutation rates that are orders of magnitude higher than those reported for DNA-based organisms. Mutation frequencies in RNA viruses typically range between 10^{-3} and 10^{-6} per site per replication (10) because of the intrinsic error rate of RNA polymerase and the lack of proofreading mechanisms. Consequently, RNA virus populations, even those initiated by a single infectious unit, are not clonal but consist of a large number of genetic microvariants referred to as quasispecies (7, 10). The high genetic variability in these quasispecies can facilitate rapid adaptation to new environments. Moreover, this variability can pose distinct clinical challenges for the treatment and prevention of diseases caused by RNA viruses. In particular, there is potential for rapid development of antiviral resistance and for the evolution of vaccine-escape mutants (6), although the latter has not proved to be an obstacle for the majority of vaccine-preventable RNA virus infections.

While the spontaneous mutation rate plays an important role in determining these population dynamics, it can be difficult to estimate mutation rates accurately. Indirect estimates based on the accumulation of mutations in field or experimental populations are often confounded by population history and natural selection. For example, recent population bottlenecks or selection for or against particular alleles often has a much greater impact on the rate of mutation accumulation than the polymerase error rate itself. Similarly, estimates derived from measures of mutant frequencies in the laboratory may also be confounded by selection and by phenotypic masking, which occurs when viruses of a particular genotype are associated with the coat proteins of a more common genotype (5). Constraints inherent in these methods can lead to over- or underestimates of the mutation rate by large factors and may explain some of the variability in reported estimates for particular species (5).

A recent series of carefully designed studies focusing on two nonsegmented RNA viruses, vesicular stomatitis virus (VSV) and poliovirus, attempted to minimize these potential sources of bias (3, 4, 11, 22). On the basis of the frequency of neutral mutants at well-characterized loci conferring either guanidine resistance or resistance to a monoclonal antibody (MAb), these studies estimated a higher mutation rate for poliovirus than previously reported; for both viruses, the average mutation rate was estimated to lie between 10^{-3} and 10^{-4} per base pair per replication.

In contrast, the mutation rate of measles virus, the next likely target for global eradication following poliovirus, remains largely unexplored. Members of the *Morbillivirus* genus, including measles virus, typically have only one major serotype and a narrow host range. In the field, measles virus has been shown to maintain high levels of genetic stability, particularly in outbreak settings (17). Additionally, a laboratory study of the accumulation of mutations in the phosphoprotein (P) gene of the Edmonston wild-type strain of measles virus after 100 laboratory passages estimated a lower mutation rate (1.4×10^{-6} per base per replication) than anticipated for an RNA virus (13). This study, however, did not control for important, potentially confounding factors, such as selection. Furthermore, the P gene, because it encodes three proteins using different reading frames of the same nucleotide sequence, is anticipated to be more stable than other portions of the measles virus genome.

Here we report a direct estimate of the spontaneous mutation rate of measles virus, based on mutations affecting the epitope of a neutralizing MAb in the hemagglutinin (H) gene.

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To avoid some of the biases of estimates derived from sequence divergence data or mutant frequency, we estimated mutation rate by the fluctuation test method, developed originally by Luria and Delbruck (14). Unlike results obtained using the above methods, fluctuation test results are not confounded by the effects of natural selection, except for the extreme case of mutations conferring total lethality. We compared our estimates with those obtained by other methods and for other RNA viruses and examined the clinical and evolutionary significance of these results.

MATERIALS AND METHODS

Cells and virus. Viruses in all assays were propagated at 37°C and 5% CO_2 on monolayers of Vero cells maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), glutamine, and antibiotics. The Edmonston wild-type strain of measles virus (obtained from P. Albrecht) was used as a stock virus, following one round of plaque purification and amplification to high titer (10^7 PFU).

Plaque assays. Vero cells were seeded into 24-well tissue culture plates (2-cm^2 wells) and grown in MEM supplemented with 10% FBS until they achieved a 90 to 95% confluency monolayer (approximately 10^4 cells). Growth medium was then removed, and 0.2 ml of an appropriate dilution of virus was allowed to adsorb to cells for 1 h at 37°C. For measles virus, this is sufficient time to allow for the majority of viruses to adsorb to cells, while greater than 20 h is typically required for production of a new burst of viral particles (1). After this adsorption period, the wells were covered with 0.5 ml of a 1% agarose overlay containing MEM supplemented with 2% FBS. When viruses were grown in the presence of MAb, this overlay was supplemented with an appropriate concentration of antibody. The addition of antibody to the overlay following viral adsorption minimizes phenotypic masking (11). Cells and viruses were then allowed to incubate at 37°C for 5 days, at which point they were stained with a 5% solution of neutral red in phosphate-buffered saline and scored for plaques.

MAb. We used an anti-H MAb, 80-III-B2, produced from mouse ascites fluid as described previously (8) and frozen in aliquots after preparation. The epitope of 80-III-B2 has been characterized previously (21), and previous studies using the expression of chimeric H proteins have identified single point mutations associated with resistance to this MAb, including aspartic acid → tyrosine at residue 505 of the H gene (strain HNT-PI) (12); aspartic acid → glycine at residue 505 (strain CAM-70) (18); and glycine → aspartic acid at residue 506 (strain S-191) (18).

Estimation of mutation rate by the null class fluctuation test method. The null class fluctuation test assay (14) (described in more detail in reference 24; see reference 5 for applications to RNA viruses) is based on the assumption that mutational events are distributed randomly across independent viral cultures according to a Poisson distribution. It involves establishing a number of initially mutant-free virus cultures (by inoculating tissue culture wells with a very small inoculum of virus), allowing them to grow in parallel until approximately half of the cultures contain at least one mutation to the phenotype of interest, measuring total viral population size in a set of representative cultures, and screening the remainder for the presence or absence of mutants. If the initial viral inoculum is small, the average number of newly produced viral particles is approximated by the total number present in each culture, n. If the mutation rate to a particular phenotype per viral replication is μ, then each viral culture should have an average of μn mutations. The proportion of cultures with no mutations (P_0, the null class) is then equal to $e^{-\mu n}$, based on the Poisson distribution. Thus, experiments measuring n and P_0 permit the direct estimation of the mutation rate, μ.

We adapted this method to the case of measles virus by focusing on mutations conferring resistance to MAb 80-III-B2. To obtain accurate estimates of P_0 and n, we performed three independent experiments containing at least 60 independent assay wells each. In each experiment, 2-cm^2 tissue culture wells covered with a virtually confluent cell layer were infected with 50 to 100 PFU of stock virus (a multiplicity of infection of approximately 0.01). Infected wells were incubated for 1 h at 37°C to allow for viral adsorption, and the cells were grown at 37°C in 1 ml of MEM containing 2% FBS for 36 h. This period was determined in a series of pilot studies to be sufficient for the appearance of MAb-resistant mutants in approximately 50% of the cultures. After 36 h, all but 250 μl of MEM was aspirated and the tissue culture wells were frozen and thawed. Virus from each well was then harvested independently and transferred to a freshly seeded well, where it was screened by plaque assay for the presence of MAb-resistant mutants. Viruses from five wells per experiment were plated in the absence of MAb to estimate the final number of viruses per culture, n.

Isolation and genetic characterization of MAb-resistant mutants. Five MAb-resistant mutants observed in fluctuation tests were randomly chosen for further genetic analysis. Resistant virus was harvested by removing the contents of a well-isolated plaque with a sterile Pasteur pipette. The agarose plug was placed in 250 μl of MEM, freeze-thawed, amplified in an 2-cm^2 well in the absence of MAb, and then plaque purified and amplified two more times in the continuous presence of MAb. This procedure was necessary to ensure that wild-type virus, present in each initial plaque pick in much higher numbers than MAb-resistant virus, was reduced to negligible levels prior to genetic characterization.

TABLE 1. Fluctuation test results

Replicate	No. of virus populations	Initial viral inoculum (PFU)	Final viral no. (PFU/population)[a]	Populations with no MAb-resistant mutants	Estimated mutation rate (μ)[b]
1	68	130	1,950	52	1.4×10^{-4}
2	68	260	7,200	44	6×10^{-5}
3	91	123	7,000	27	1.7×10^{-4}

[a] Average number of viruses per population, based on titers in the absence of MAb from at least five independent populations (tissue culture wells) per replicate experiment.

[b] Estimated as described in Materials and Methods.

Viral RNA was extracted by using 8 M guanidinium hydrochloride, and reverse transcription-PCR was performed with primers that amplify the entire coding region of the H gene, as previously described (16, 19, 20). PCR products were sequenced directly by cycle sequencing in a model 373A DNA sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, Calif.).

RESULTS

Concentration of MAb required to neutralize virus. The concentration of MAb required for complete neutralization of virus was determined by a plaque assay, using a series of viral dilutions and two MAb dilutions (1/200 and 1/100) in the agarose overlay. The lower concentration prevented plaque formation for viral numbers less than 8,000 PFU, while the 1/100 dilution of MAb prevented formation of any visible plaques for virus numbers less than 80,000 PFU. Consequently, a 1/100 dilution of MAb was always used when antibody was required, and viral numbers per tissue culture well were kept well below the maximum neutralizable number at that concentration. At a 1/100 dilution, MAb-resistant viruses were only slightly inhibited by the presence of antibody; this inhibition resulted in a slightly smaller plaque size relative to that of wild-type viruses and a slightly slower progression of plaque formation but did not alter estimated titers after 5 days of incubation at 37°C.

Frequency of MAb-resistant mutants in the initial viral stock. Because the viral stock used in these experiments was of a high titer (10^7 PFU/ml) and therefore was expected to contain some resistant mutants, we measured the frequency of resistant mutants present in the initial stock by a plaque assay. Forty-five replicate 2-cm^2 wells coated with Vero cells were infected with an average of 8,400 infectious units each (a multiplicity of infection of approximately 0.8) and incubated for 5 days in the presence of an antibody-containing agarose overlay. Across wells, a total of 117 PFU were observed. Thus, MAb-resistant mutants were present in the stock at a rate of approximately 1/3,000 PFU.

Fluctuation test results. The results of three replicate fluctuation test experiments are shown in Table 1. Initial inoculum sizes of 100 to 200 PFU per population were small enough to make it likely that populations did not include MAb-resistant variants at the onset. Populations were considered to contain no MAb mutants at the end of the experiment if they produced no detectable plaques when a plaque assay was performed in the presence of MAb and showed no detectable cytopathic effect at 10× magnification. Populations that did show evidence of MAb-resistant mutants often had low numbers of visible plaques (<5), and only very rarely did we observe populations that contained large numbers of resistant mutants. There was some variation in results between replicate experiments. This could have been due in part to a slight variation in starting conditions between replicates. In particular, plaque detection was more difficult when the Vero cell monolayer was more dense at the time of infection. Based on the results in

TABLE 2. Point mutations distinguishing MAb-resistant clones (n = 5) from the MAb-sensitive wild-type strain

H gene amino acid	Base substitution	Coding change	No. of mutants
505	GAT→AAT	D→N	2
507	GAT→TAT	D→Y	1
507	GAT→GGT	D→G	1
530	GAT→GGT	D→G	1

Table 1, the average mutation rate estimated from these experiments was $1.2 \times 10^{-4} \pm 2.7 \times 10^{-5}$ per replication.

Genetic characterization of MAb-resistant mutants. The entire H gene of each of five independently isolated MAb-resistant mutants was sequenced, along with the H gene of the wild-type (MAb-sensitive) stock virus. The H gene of the stock strain matched that of previously reported sequences for the Edmonston wild type. In contrast, the H genes of the MAb-resistant mutants each differed from the wild type by a single base substitution. The observed point mutations and their respective coding changes are shown in Table 2.

DISCUSSION

Previous studies estimating the mutation rate in RNA viruses have typically reported rates of between 10^{-4} and 10^{-5} per site per replication (10). The average estimate that we obtained for measles virus, 1.2×10^{-4} per replication, falls in this range. This mutation rate, however, represents a composite of the rates of all mutations that conferred resistance to MAb 80-III-B2. Genetic characterization of five isolated resistant mutants demonstrated that single point mutations that occurred at any one of four bases in the H gene were associated with this phenotype (Table 2). Therefore, to calculate the mutation rate per nucleotide site per replication, we multiplied the above mutation rate by 3 (there are three possible unique base substitutions at any given site) and divided by 4 (the number of bases where mutations occurred), obtaining an estimate of 9×10^{-5} per nucleotide site per replication. This assumes, as a best approximation, that substitutions are equally likely for all four nucleotides and that rates of substitution do not vary significantly between base pairs (5). This is not always valid; in particular, in RNA viruses, transitions are often more common than transversions (10). Because the MAb resistance phenotype we investigated was caused either by transitions or by transversions (Table 2), the rate we estimated is more likely to reflect an average rate than estimates based on only one class of substitution.

We further estimated a genomic mutation rate of 1.43 per replication by multiplying the mutation rate per base pair by the genome size of measles virus (15.9 kb [9]). This again is similar to the range of genomic mutation rates estimated for poliovirus (0.475 to 1.21) and slightly lower than estimates for VSV (2.75 to 4.28) (5). We note, however, that because it is not possible to estimate mutation rates at every site within a genome, estimates of the genomic mutation rate assume that what is true for particular sites of the genome in particular viral clones is true of the species as a whole. Because of the quasispecies nature of RNA viruses and evidence that mutation rates at different sites of the genome may vary (10), this assumption may not always hold. Nonetheless, our data suggest that, like other RNA viruses, measles virus appears to have a mutation rate close to the maximum tolerable deleterious mutation rate that cannot greatly exceed 1 per genome per replication (5).

Because we used the null class fluctuation test method of estimating mutation rate, population history did not bias outcomes in our assays and the effects of selection were minimized. Furthermore, by allowing virus to adsorb to cells in the absence of MAb prior to screening for MAb resistance, we minimized the opportunity for phenotypic masking by wild-type virus, a problem that can lead to large underestimates of mutation rate (11). Because our experimental design required us to isolate MAb-resistant mutants from a dense lawn of wild-type virus, however, multiple rounds of plaque purification and amplification in the presence of the MAb were in some cases required to dilute out wild-type virus. For two of the five mutants analyzed, resistance mutations could be detected by sequencing after one round of plaque purification. For the remaining mutants, in contrast, sequencing after one round of plaque purification revealed the wild-type sequence, despite a clearly resistant phenotype; mutations were identified by sequencing only after two additional rounds of plaque purification in the presence of MAb.

Consistent with results of previous studies (12), the genetic sequence of the entire H gene of five randomly chosen MAb-resistant mutants confirmed that a single point mutation is sufficient to confer the resistance phenotype. The sequence analysis revealed, however, that these single point mutations did not all occur at the same site within the H gene. In addition to mutations at residue 505, which have been identified previously (12), we identified mutations associated with resistance at two locations in residue 507 and also a mutation in the codon for amino acid 530 (Table 2). Residue 530 has not been shown previously to be associated with the binding of MAb 80-III-B2. Radioimmunoprecipitation assays confirmed, however, that this point mutation prevented the MAb from binding to the H protein (25). Because we sequenced the H gene of only five MAb-resistant mutants, it is possible that point mutations at bases other than the four we identified conferred the resistance phenotype in some of the uncharacterized mutants. Furthermore, Western blot analysis showed that the MAb did not bind to the wild-type H gene under denaturing conditions, suggesting that the epitope was not linear and that mutations affecting the epitope could occur outside residues 505 and 506. This does not affect our estimate of the phenotypic mutation rate, but it could lead to overestimates of the mutation rate per base pair and per genome.

The spontaneous mutation rate of measles virus that we obtained in this study provides an estimate of the error rate of measles virus RNA polymerase. Additional studies of mutation rates in other measles virus strains and at different sites within the measles virus genome will add generality to these results. While we anticipate that a similar error rate operates under natural conditions, we cannot compare our results directly with those of studies that have measured the rate of accumulation of mutations in field isolates. The primary obstacle to this comparison is that a multitude of factors, in addition to the inherent error rate, can influence the accumulation of mutations, and it is virtually impossible to control for these factors in the field. For example, the hemagglutinin gene of influenza A virus has been found to accumulate mutations more rapidly in human lineages (7.9 nucleotide substitutions per year) than in equine lineages (3.1 nucleotide substitutions per year), even though the intrinsic RNA error rate is not anticipated to vary significantly in these two hosts (2). Similarly, controlled laboratory studies of VSV have shown that under some conditions (for example, during dilute passages as opposed to high-multiplicity passages or persistent infections), virus populations maintain high levels of genetic stability (10, 23). Unfortunately, teasing apart the factors resulting in slow or rapid

rates of mutation accumulation in natural environments is difficult, and genetic bottlenecks or selection at the site of a mutation or related loci can have a much stronger impact on observed genetic variability than the polymerase error rate itself.

Thus, the high mutation rate we estimated for measles virus, in line with those reported for other RNA viruses, does not necessarily imply rapid evolution in natural measles virus populations. In fact, observations that field isolates collected at different times often show very low levels of nucleotide divergence (15, 16) suggest that under some conditions measles virus populations do not evolve rapidly. Myriad factors could contribute to this stability, including the lack of recombination in morbilliviruses, strict constraints on insertions and deletions, the limited host range of measles virus, and functional constraints due to measles virus's protein receptor. In the context of measles virus elimination efforts, evidence for a high mutation rate suggests that the possibility of strains that may escape neutralization by vaccine must be considered, although to date there is no evidence of such vaccine-escape mutants. From an evolutionary perspective, further investigation into forces maintaining the genetic stability of measles virus in the field will contribute substantially to an understanding of measles virus population biology.

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