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Sequence divergence of measles virus haemagglutinin during natural evolution and adaptation to cell culture

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Phylogenetic analysis of the sequence of the H gene of 75 measles virus (MV) strains (32 published and 43 new sequences) was carried out. The lineage groups described from comparison of the nucleotide sequences encoding the C-terminal regions of the N protein of MV were the same as those derived from the H gene sequences in almost all cases. The databases document a number of distinct genotype switches that have occurred in Madrid (Spain). Well-documented is the complete replacement of lineage group C2, the common European genotype at that time, with that of group D3 around the autumn of 1993. No further isolations of group C2 took place in Madrid after this time. The rate of mutation of the H gene sequences of MV genotype D3 circulating in Madrid from 1993 to 1996 was very low (5×10^{-4} per annum for a given nucleotide position). This is an order of magnitude lower than the rates of mutation observed in the HN genes of human influenza A viruses. The ratio of expressed over silent mutations indicated that the divergence was not driven by immune selection in this gene. Variations in amino acid 117 of the H protein (F or L) may be related to the ability of some strains to haemagglutinate only in the presence of salt. Adaptation of MV to different primate cell types was associated with very small numbers of mutations in the H gene. The changes could not be predicted when virus previously grown in human B cell lines was adapted to monkey Vero cells. In contrast, rodent brain-adapted viruses displayed a lot of amino acid sequence variation from normal MV strains. There was no convincing evidence for recombination between MV genotypes.

Introduction

Measles virus (MV) is an important pathogen that kills approximately 1·2 million children per annum and causes the highest mortality of all vaccine-preventable virus diseases. The virus is monotypic and the vaccines, which are derived from a single 1954 isolate (Rota *et al.*, 1994), still provide protection against all the virus genotypes that are circulating at present. Tamin *et al.* (1994) have demonstrated that the neutralization titres of sera from human vaccinees are lower against some of the wild-type viruses than against the vaccine strains. Thus, it is important to assess whether the divergence of various measles isolates may in the longer term lead to a failure of the current vaccines to protect against disease induced by wild-type viruses. Hence, it is important to monitor MV variation.

The existence of at least eight genotypes of MV has been determined by analysis of the most variable coding sequence in the genome, namely that which encodes the last 151 amino acid residues of the nucleocapsid (N) protein (Taylor *et al.*, 1991; Rima *et al.*, 1995*a*). The biological significance of this variation is not known but as the N protein provides a very powerful stimulus to the immune system in the acute phase of the disease, these sequence differences may reflect immunological pressure. The other major antigen that elicits immune responses, probably primarily in the form of neutralizing antibodies, is the viral haemagglutinin (H) glycoprotein. The neutralization titre of a given serum sample has been shown to correlate well with the degree of protection against virus disease and the neutralizing ability of maternal antibody is

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Table 1. Strain derivations and sequence references

Group	Abbreviation	Description	Sequence reference	Accession number
A	Edm wt	Edmonston wild-type/USA/1954	Rota *et al.* (1994)	U03669
	Edm vc	Edmonston vaccine	Alkhatib & Briedis (1986)	M14877
	Mor	Moraten vaccine	This work	Z80791
	Edm P9	Salt-dependent Edmonston vaccine	This work	Z80793
	CAM/70	CAM vaccine	Rota *et al.* (1994)	U03649
	CAM/RB	Rat brain-adapted CAM	Liebert *et al.* (1994)	Z80792
	Schw	Schwarz vaccine	Rota *et al.* (1994)	U03667
	Chg	Changchun-147 vaccine/China/1957	Rota *et al.* (1994)	U03652
	Len	Leningrad-16/Russia/1960	Rota *et al.* (1994)	U03660
	Zag	Edmonston-Zagreb vaccine	Rota *et al.* (1994)	U03671
	S19	Shanghai vaccine/China/1960	Rota *et al.* (1994)	U03663
	AIK-C	Edmonston-derived vaccine	Mori *et al.* (1993)	S58435
	Hal	Hallé 'SSPE' isolate/USA/1971	Gerald *et al.* (1986)	X04720
	Hu2	Schwarz vaccine-associated case/N Ireland/1974	This work	Z80790
	Phil	Philadelphia-26/USA/1970	Rota *et al.* (1994)	U08417
	Cov77	Coventry-77/England/1993	Outlaw & Pringle (1996)	NA
	Cov41	Coventry-41/England/1993	Outlaw & Pringle (1996)	NA
	Cov49	Coventry-49/England/1993	Outlaw & Pringle (1996)	NA
	Cov81	Coventry-81/England/1993	Outlaw & Pringle (1996)	NA
	Cov105	Coventry-105/England/1993	Outlaw & Pringle (1996)	NA
	HNT	Hamster neurotropic/Philadelphia-26	Hummel *et al.* (1994)	U08414
	HNTpi	HNT pers. inf./Philadelphia-26	Hummel *et al.* (1994)	U08411
C1	SIP3A	IP3CA SSPE cell line/USA/early 70s	Cattaneo *et al.* (1989)	X16566
	S(A)	SSPE case A/Germany/mid 80s	Cattaneo *et al.* (1989)	X16567
	Ya	Yamagata SSPE/Japan/late 80s	Komase *et al.* (1990)	NA
	Sma81	SSPE Madrid/Spain/1981	This work	Z80810
	Mad79	Wild-type Madrid/Spain/1979	This work	Z80831
	SBI	Wild-type Bonn/Germany/1992	This work	Z80806
	S(K)	SSPE case K/Germany/mid 80s	This work	Z80807
	EBL	Wild-type lymphocyte/Japan/1985	This work	Z80803
	EBT	Wild-type throat wash/Japan/1985	This work	Z80802
C2	JM	Wild-type/USA/1977	Rota *et al.* (1992)	M81898
	S(B)	SSPE case B/Austria/mid 80s	Cattaneo *et al.* (1989)	X16568
	Bil	Wild-type Bilthoven/The Netherlands/1991	This work	Z80796
	WTFb	Erlangen BJAB/Germany/1992	This work	Z80808
	WTFv	Erlangen Vero/Germany/1992	This work	Z80809
	DLb	Wild-type DL BJAB/Germany/1992	This work	Z80800
	DLv	Wild-type DV Vero/Germany/1992	This work	Z80801
	DA	Wild-type/Germany/1992	This work	Z80799
	AB	Wild-type AB/Germany/1993	This work	Z80794
	AC	Wild-type AC/Germany/1993	This work	Z80795
	ADM	Wild-type Madrid/Spain/1992	This work	Z80811
	RMS	Wild-type Madrid Ma92R/Spain/1992	This work	Z80812
	JMC	Wild-type Madrid/Spain/1992	This work	Z80914
	FV	Wild-type Madrid Ma92F/Spain/1992	This work	Z80813
	TAM	Wild-type Madrid/Spain/1992	This work	Z80915
D1	MVO	Wild-type Bristol/England/1974	This work	Z80804
	MVP	Wild-type Bristol/England/1974	This work	Z80805
D2	Bo83	Wild-type Boston/USA/1983	This work	M81897
	Chi1	Wild-type Chicago-1/USA/1989	Rota *et al.* (1992)	M81895
	Chi2	Wild-type Chicago-2/USA/1989	Rota *et al.* (1992)	M81896
	SND	Wild-type San Diego/USA/1989	Rota *et al.* (1992)	M81902
	CL	Wild-type Birmingham/England/1988	Schulz *et al.* (1992)	NA
	SE	Wild-type Birmingham/England/1988	Schulz *et al.* (1992)	NA
	TT	Kawasaki case/England/1991	Schulz *et al.* (1992)	NA

Table 1. *Cont.*

Group	Abbreviation	Description	Sequence reference	Accession number
D3	AGR	Wild-type Madrid/Spain/1993	This work	Z80818
	CGL	Wild-type Madrid/Spain/1994	This work	Z80822
	BCL	Wild-type Madrid Ma94B/Spain/1994	This work	Z80819
	FPM	Wild-type Madrid/Spain/1994	This work	Z80824
	ISL	Wild-type Madrid/Spain/1994	This work	Z80826
	ABP	Wild-type Madrid/Spain/1994	This work	Z80816
	ACH	Wild-type Madrid/Spain/1994	This work	Z80817
	MCR	Wild-type Madrid/Spain/1994	This work	Z80827
	C-M	Wild-type Madrid/Spain/1994	This work	Z80821
	BPG	Wild-type Madrid/Spain/1994	This work	Z80820
	DGL	Wild-type Madrid/Spain/1994	This work	Z80823
	RMB	Wild-type Madrid/Spain/1995	This work	Z80829
	IFF	Wild-type Madrid/Spain/1995	This work	Z80825
	LMP	Wild-type Madrid/Spain/1996	This work	Z80832
E	Lec	Lec SSPE/USA/1970	Hu *et al.* (1993)	X68043
	Brx	Encephalitis case/Germany/1971	This work	Z80797
	CM	Wild-type/USA/late 70s	This work	Z80798
	S(C)	MIBE case/USA/late 70s	Cattaneo *et al.* (1989)	X16569
F	SMa 79	SSPE case/Spain/1979	This work	Z80828
	SMa 94	SSPE case/Spain/1994	This work	Z80830

NA, Not available.

considered of prime importance in conferring resistance to virus infection and vaccination in new-born infants. However, the neutralization titre in adult sera is considered to be only a surrogate correlate to protection as T cell-dependent mechanisms are probably more important in the clearance of the virus than humoral responses (reviewed by Griffin & Bellini, 1996). Here, we analyse 32 published (see Table 1) and 43 unpublished sequences of the H gene of 49 MV isolates, 11 subacute sclerosing panencephalitis (SSPE) patient-derived sequences and 16 derived strains, mostly from the Edmonston isolate. A main objective was to determine whether the genotypes established on the basis of the C-terminal 456 nucleotides of the N gene are the same as those resulting from an analysis of the H gene sequences of the same isolates. The present study was also designed to assess whether any evidence could be found for recombination in MV and what rate of nucleotide substitutions is observed in a number of data sets of repeated virus isolations made from one genotype in a single location. Repeated isolation of MV in Madrid (Spain) has allowed the documentation of a number of very abrupt genotype switches most likely due to importation of new virus strains from elsewhere.

Methods

■ **Virus strains.** The acute measles viruses from Madrid patients were isolated from throat swabs, except for isolates FPM, ISL and IFF, which were obtained from peripheral blood mononuclear cells, in marmoset B lymphoblastoid B95-8 cells. Five to seven days after primary inoculation, cultures showing syncytia formation were centrifuged, cell pellets and supernatants were collected and aliquots frozen at −80 °C. Brain necropsia samples from Madrid SSPE patients were immediately frozen at −80 °C or immersed in liquid nitrogen.

■ **RT–PCR, cloning and nucleotide sequence determinations.** RNA was extracted from Madrid MV isolate-infected cells or SSPE brain cells using the guanidinium isothiocyanate technique. MV H gene-specific cDNA was synthesized using mouse Moloney virus reverse transcriptase and amplified by PCR with the following primers:
F34, 5′ TGTCTTGGAGGGTTGATAGG 3′
L97, 5′ CACTGACCTCATCTCAGTCTTGACT 3′
H181, 5′ TTAGGGTGCAAGATCATCCACA 3′
H172, 5′ TTCTGATGTCTATTTCACACTAGTGGGT 3′
PCR products were sequenced directly in both directions by the Sanger method with primers constructed at 400–500 base intervals along the H coding sequences, based on the sequence of the constructed Edmonston strain of MV (Alkhatib & Briedis, 1986).

The fresh isolates from Germany were propagated on BJAB cells or adapted to Vero cells. Infected-cell RNA was reverse-transcribed and cloned as described earlier (Baczko *et al.*, 1991). The methodology used for the determination of the sequences of the H genes of various virus isolates was as described before for N gene sequences (Rima *et al.*, 1995*a*).

■ **Viruses and sequences.** The sequences described in this paper which have been published earlier are referenced in Table 1. This table also gives the accession numbers for these sequences and those determined during the course of the current study. The sequences have been grouped by genotype where these were determined unambiguously.

■ **Phylogenetic analysis.** Phylogenetic analysis was performed using the CLUSTAL W software mounted on the seqnet at Daresbury, UK. Bootstrap trials (1000) were made and the resulting unrooted tree was drawn as before for the C-terminal part of the N gene of various MV strains (Rima *et al.*, 1995*a*).

Results and Discussion

Genotypes established on the basis of the H gene sequences

Fig. 1 shows an unrooted tree based on the neighbour-joining methodology. The grouping of various MV strains is similar to that calculated on the basis of the N gene sequence of MV (Rima *et al.*, 1995*a*). However, significant changes in the arrangement of the central nodes of the tree are evident. As these are very susceptible to the complement of sequences included and are not very well supported in the bootstrap analysis it is clear that the arrangement of nodes in the centre of the diagram is neither definitive nor informative (Rima *et al.*, 1995*a*). Furthermore, the original C1 group determined on the basis of the N gene sequences contained the SIP3A sequence which, as far as the N gene is concerned, is very similar to the others belonging in the group. However, the H gene of this SSPE isolate has undergone biased hypermutation (Cattaneo *et al.*, 1989) and this disturbs the arrangement within the C1 group. The groupings have been used to construct Table 1 which describes the origin of strains and sequences with references as appropriate. No new groups of wild-type isolates were found in this analysis. The analysis of the H gene groups the strains of genotypes A, E and F the same as before. No H sequences of groups B1 and B2 were analysed in this study, but quite recently it was reported that genotype B strains of African origin also formed a distinct genotype when H gene sequences were analysed (Rota *et al.*, 1996). These data again confirm that the grouping of MV strains into different genotypes is the same when either the N or H gene sequences are compared. The new wild-type isolates from Germany and Spain primarily fall in the old groups C2 and D3 established from the N gene tree and these extend the number of strains in these groups significantly.

Recently, Outlaw & Pringle (1996) reported a number of H gene sequences of isolates made in Coventry (UK) in 1993. These sequences were interesting in that several contained an in-frame premature stop codon which led to deletion of 35 amino acid residues at the C terminus of the H protein. In the nucleotide sequence analysis the group of wild-type isolates from Coventry seems to be derived from lineage group A viruses, i.e. the virus lineage from which all vaccines are derived. One isolate (Cov77) only showed two mutations from the Edmonston strain sequence and the others showed the same eight mutations from the Edmonston strain. It is not clear at present whether these isolates are vaccine-derived or represent wild-type viruses of group A. The last street isolations of this genotype were made in 1970. It would be interesting to determine the nucleotide sequences of the N genes of the Coventry isolates so as to establish their relationship to the other MV lineages more precisely.

In the tree shown in Fig. 1 we were also able to demonstrate that the Lec strain of MV (Hu *et al.*, 1993), isolated in the USA in 1970 from an SSPE case, belongs to group E. This is in line with the two other isolates in this group, which are North American, but not with the Braxator (Brx) strain isolated in Germany. The Lec strain represents the only non-defective SSPE patient-derived isolate that does not belong to group A. The Yamagata-1 (Ya) SSPE strain is partially defective and propagated as an infected culture (Komase *et al.*, 1990). In a previous paper (Rima *et al.*, 1995*a*) we suggested that the lack of evolution in group A indicated that the non-defective SSPE isolates are probably laboratory contaminants. However, in the case of the Ya and Lec strains, this appears not to be the case as they represent early isolates in lineage groups C1 and E, respectively. The isolation of non-defective viruses from SSPE patients' brain tissues should, however, not be surprising as recently (Baczko *et al.*, 1993) both wild-type progenitor M and hypermutated M gene sequences were derived from RNAs extracted from the same tissue blocks of the brain of an SSPE patient [case B; S(B)].

Genotype switches in Spanish isolates

The extensive numbers of isolates from Madrid (Spain), obtained from both SSPE patients and uncomplicated cases of measles, indicated that several genotype switches have occurred there since the late 1960s.

The SSPE sequences derived from patients with a documented original acute infection before 1970 indicate that at the time genotype F was prevalent. At an undetermined time point around 1970, this was replaced with viruses from the C1 group represented by a single SSPE case sequence (SMa81) and a wild-type isolate Mad79 made in 1979. In April 1992, the common European lineage group C2 became prevalent in Spain and was isolated until July 1993, to be replaced by viruses from the D3 group since October 1993. This coincided with the late phase (past the peak of 1992) of an epidemic lasting from 1990 to 1993 and the appearance of a trough in the number of cases in the period 1994–1995.

The data reported here allow an accurate description of this genotype switch. Between July and October 1993 the genotype changed completely with the introduction of an MV strain with the D3 genotype which differed in 50 nucleotide positions from the C2 group. The extent of these differences suggests that a new genotype was imported from elsewhere and took over the circulating virus, as has been reported recently in a United States measles epidemic (Rota *et al.*, 1996). There was no evidence of co-circulation of the two virus strains in the Spanish population up to the end of January 1996, but the isolation record may not be comprehensive enough to conclude this categorically, even though this series of MV isolates is unparalleled in terms of frequency of isolation of MV

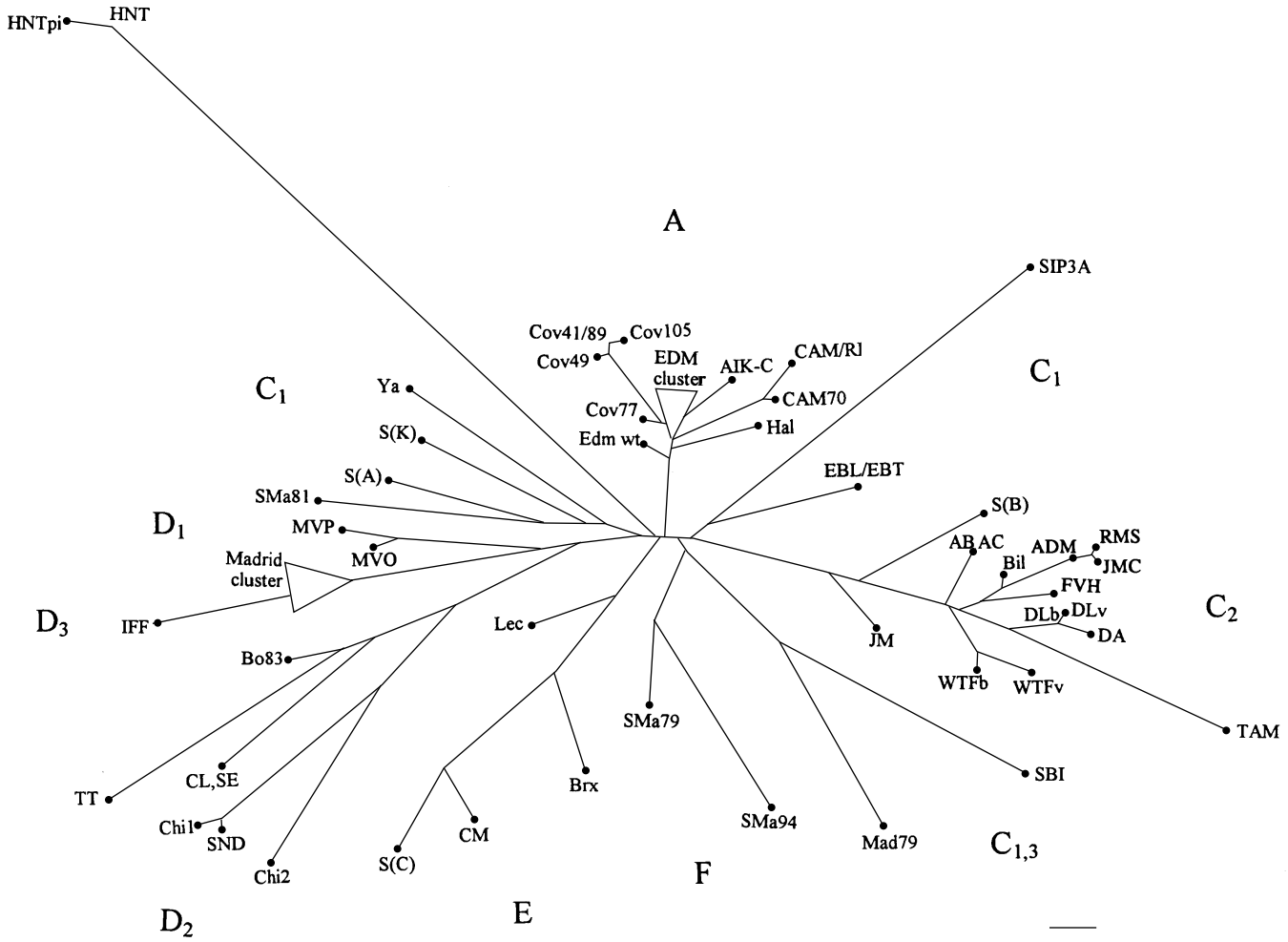

Fig. 1. Unrooted tree drawn on the basis of a CLUSTAL W analysis of the nucleotide sequences of 75 MV strains. Abbreviations of strains are given in Table 1. The genotypes are indicated in capital letters. The Edm cluster contains the strains Mor, Edm P9, Schw, Chg, Len, Zag, S19, Hu2 and Phil. The Madrid cluster contains the strains AGR, CGL, BCL, FPM, ISL, ABP, ACH, MCR, C-M, BPG, DGL, RMP and LMP. The horizontal bar indicates four nucleotide changes.

in a single location. The data show that at least four genotype changes have occurred in Spain since the late 1960s. It is not clear at present whether this relates to the geographic position of the country, on the gateway between Europe and Africa, or whether these genotype switches occur as a result of importation of new strains of MV into a country in which strains of the previously circulating genotype have become 'extinct' locally.

It is now clear that high vaccination coverage may lead to a temporary eradication of MV in a given country but that reimportation leads to renewed outbreaks in the susceptible population (Rota *et al.*, 1996). It would be interesting in this respect to determine the origin of the Coventry 1993 isolates of genotype A. They either represent possibly a vaccine-derived virus strain or reimportations from elsewhere, if this lineage is still circulating in an area of the world not under current surveillance.

Mutation rates of MV H gene sequences in epidemics

In Germany a number of isolates were made from two epidemics in 1992 (AB, AC) and 1993 (DLv/DVb, DA) belonging to the C2 lineage group, the common European lineage group that has been found to circulate in several continental countries. The 1990 isolate WTF also belongs to this group (Fig. 2). The two 1993 isolates are identical; the 1992 isolates vary by three nucleotides. Between 1990 and 1993, 12 mutations accumulated in the H gene of these isolates assuming that the 1990 sequences represent the isolates circulating in Germany in that year. The assumption that a single isolate will be representative is not necessarily valid as illustrated in the 1993 isolations of two viruses which differed in three positions. The corresponding mutation rate derived from this small data set is 2×10^{-3} per annum at each nucleotide position in the H gene. This rate is high and not found in the more extensive series of isolates made in Madrid.

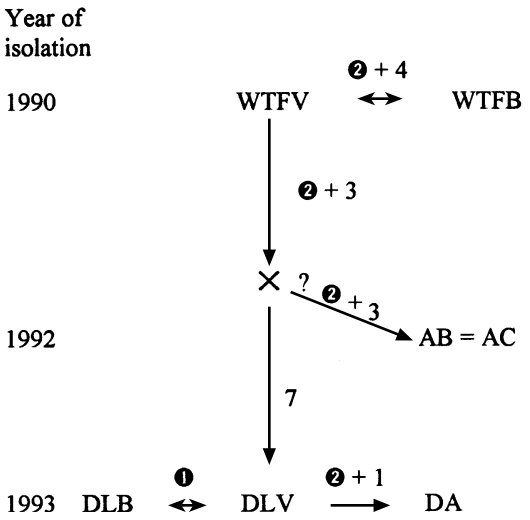

Fig. 2. Relationships between MV isolates of the C2 genotype in Germany (1990–1993). Numbers of expressed (circled) and silent (uncircled) mutations are shown. Nucleotide changes between WTFV and WTFB, and between DLV and DLB are given in Table 2.

The Spanish isolates belonged to the C2 and the D3 groups. These isolates allowed us to make the first estimates of mutation rates in the H gene of MV during an epidemic. The calculations are based on the assumptions that neither reverse transcription nor PCR induce sequence variation. The first assumption is standard and not easily amenable to verification, although our experience and that of others (Schulz *et al*., 1992) with direct RNA sequencing has indicated that it is probably correct. The second assumption about PCR has been verified by sequencing a number of clones so that the sequence reported for each virus isolate from Germany is an average from four or five clones. Possible errors of RT–PCR have been minimized in the case of the Spanish isolates by direct sequencing of PCR products. Based on these assumptions the rate of mutation in the Spanish C2 isolates from 24 April 1992 (RMS) to 7 March 1993 (JMC) is a single nucleotide and thus can be expressed as 5×10^{-4} per annum. However, as indicated in Fig. 3, two isolates from this group, FVH (from 17 March 1993) and TAM (14 July 1993) indicate that between 21 May 1992 and March 1993 a new virus strain had evolved in which at least six nucleotides had changed, giving rise to the two isolates FVH (with a further four mutations) and TAM (with a further seven mutations). These data suggest a mutation rate of 6×10^{-3} per annum. This is probably an overestimate of the true rate but it is questionable whether this can be established from these data and this isolation record. The relationships within the second series of isolates of the D3 group in Spain are indicated in Fig. 4 which probably provides a more complete representation of the variation of the virus during an epidemic. From 15 October 1993 until 25 June 1994 identical strains were isolated (series AGR–CGL – FPM – ISL – C-M), but over the same period isolates with single and double mutations were also obtained.

The mutation rate calculated from these data is 4×10^{-4} per annum. However, in May 1995 a virus from this lineage group was isolated with 11 mutations (IFF). Since the recent January 1996 isolate (LMP) had only one mutation with respect to the original AGR sequence, it appears that the IFF sequence isolated in 1995 did not get fixed in the population. This series of isolates thus indicates that the gene sequences of the virus can be remarkably stable over several years but that sudden variants occur in the population which contain a large number of mutations.

In relation to the mechanism that underlies this variation it is important to note that in the mutations observed in the C2 group 3 were expressed and 15 mutations were silent. The ratio of expressed over silent mutations (e/s) is thus 0·2 for this data set. In the D3 group there were 7 expressed and 14 silent giving an e/s ratio of 0·5. Both values would be indicative of random drift rather than selection for amino acid sequence changes in the H protein, possibly driven by the immune system, as might be expected when MV circulates in a partially protected population. It is also noteworthy that in the D3 group there are no specific mutations that are fixed during this period and all variations from the AGR isolate represent unique changes in the H gene. Also, none except the aforementioned IFF isolate had more than one expressed mutation. This again indicates that specific, immune-driven mechanisms are not likely to cause the gradual accumulation of mutations in the H gene of MV.

It thus appears that at least in the Spanish series of isolates, mutation rates for the H gene of MV are extremely low. Major changes are associated with the introduction of completely new genotypes which remain stable for periods of more than 2 years. The mutation rates in the C2 and D3 groups suggest a rate of nucleotide replacement of approximately 4×10^{-4}. This is at least an order of magnitude smaller than the rates observed in the HN protein of human influenza A viruses during epidemics with the various HN types, which range from $5{\cdot}8–17 \times 10^{-3}$ and for the NA protein at $3{\cdot}2 \times 10^{-3}$ (Domingo & Holland, 1994). The data thus support the notion that MV is an extremely stable virus which, during epidemics, only alters very slowly. These mutation rate estimates would also infer that the various genotypes presently found circulating in the world reflect long-standing variation of MV. An alternative argument is that they have resulted from sudden spurts of mutation as observed for example in the case of the IFF isolate becoming fixed in geographically isolated populations. It is impossible to distinguish between these two alternatives at present but the fact that representatives of clearly distinguishable genotypes have been isolated over long periods would favour the former explanation. A recent paper by Rota *et al*. (1996) also stresses the point that variation between strains in a given genotype is small ($< 0{\cdot}5\,\%$).

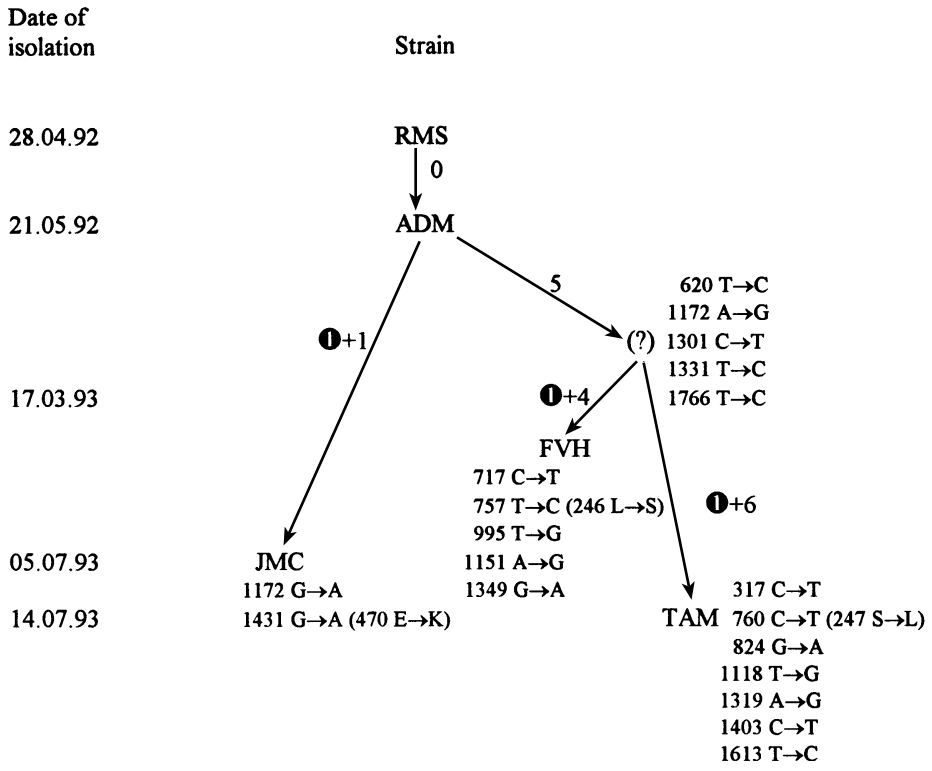

Fig. 3. Relationships between MV isolates of the C2 genotype in Spain (28.04.92–14.07.93). Numbers of expressed (circled) and silent (uncircled) mutations are shown.

Adaptation to cells of different tissues

The data presented in Table 2 consist of sequences of three pairs of viruses adapted to different cell lines. The WFT virus isolated in 1990 from an acute case of MV infection in Germany in BJAB cells (a human B cell lineage) was adapted to Vero cells (African green monkey kidney cells). Changes between the two viruses were restricted to six nucleotide changes in the WTFv (Vero cell-adapted) compared to the WTFb (BJAB-adapted) strains. Of these two are expressed (Table 2) and four are silent. In a similar experiment, the DL virus isolated in Germany in 1992 was maintained originally on BJAB cells and later adapted to Vero cells. In the DLb/DLv pair only one expressed mutation occurred at position 1656 (A → G) leading to a mutation at position 546 (S → G) in the H protein. This mutation is also found in the WTFv/WTFb pair (Table 2). The significance of this variation is not clear. Glycine residues are also found in this position in several other strains belonging to different lineage groups but these mutations are not genotype-specific. Thus, for example, the Edmonston wild-type (our sequence) but not the vaccine, the Hallé SSPE isolate of genotype A and the Chi1 isolate (genotype D2), but not SnD and Chi2, have the same A to G mutation and the deduced replacement of the serine residue at position 546 by glycine. The same is true for the IP3CA and Yamagata SSPE isolates in lineage group C1. Interestingly, this is also one of the three mutations that separate the HNT and HNTpi strains (Hummel *et al.*, 1994). The latter has been adapted to Vero cells after passage in mouse brain. It was also found to occur during passage of one of three fresh Japanese isolates which from the published amino acid sequences belong to genotype D2 (Shibahara *et al.*, 1994). It is difficult to conclude anything from this observation but the occurrence of this mutation in several genotypes may indicate that this residue plays a role in the adaptation of the virus to different growth conditions. Whether this is causative or an epiphenomenon requires assessment by the use of the recently developed reverse genetic system (Radecke *et al.*, 1995). A third pair-wise comparison made to study the effect of adaptation on the sequence of the H gene of MV allowed comparison of the EB strain of MV isolated in Japan in 1985 from throat washes (EBT) and lymphocytes (EBL) (Sakaguchi *et al.*, 1986). The two sequences were found to be identical. These data agree with those of Schulz *et al.* (1992), who determined the nucleotide sequences of viruses adapted to Vero cells and also of RNA directly from tissues from which the virus had been isolated and found no differences.

These adaptations all involve primate cells and tissues. Adaptation to rodent brain has been associated with substantial changes in the H protein of the CAM strain (Liebert *et al.*, 1994). Of 11 mutations, 10 were expressed, i.e. the *e/s* ratio was 10, indicating selective pressure for changes in the primary amino acid sequence of the H protein. The HNT strain

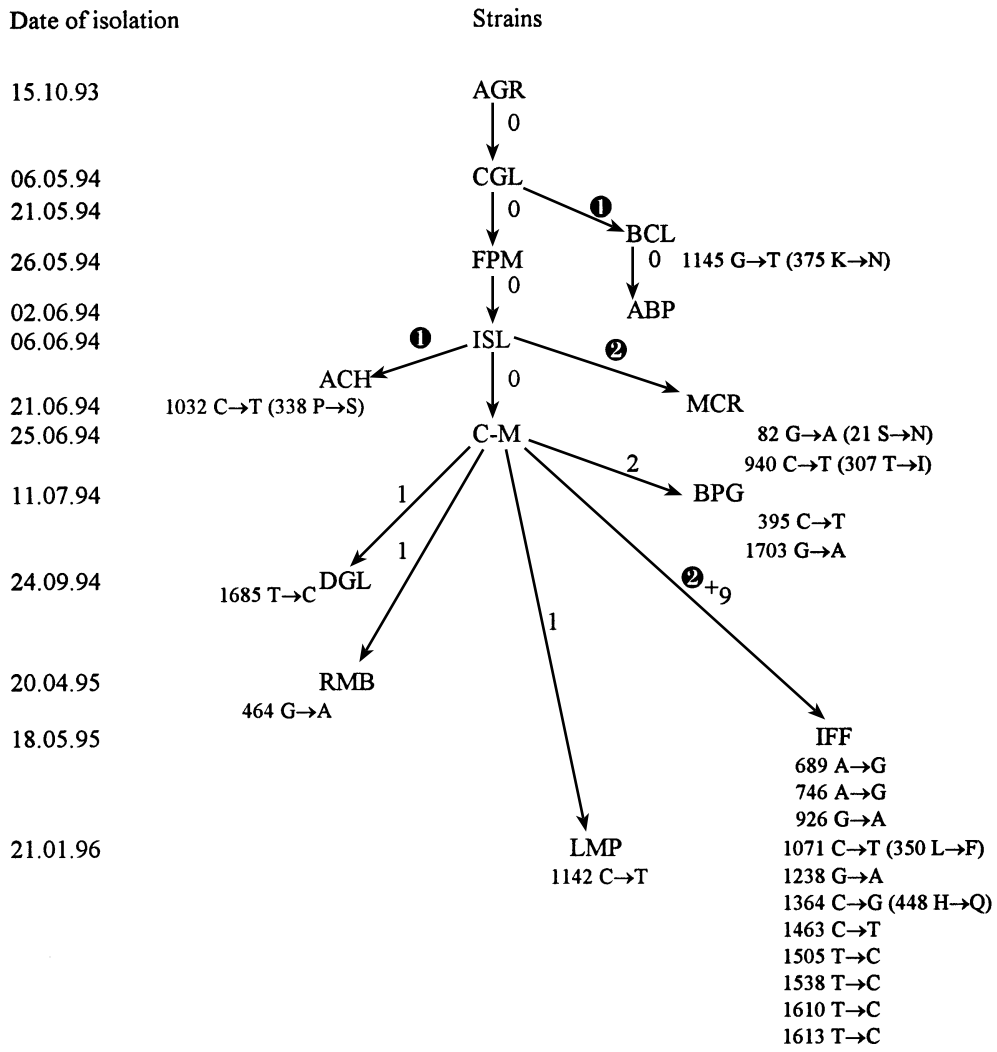

Fig. 4. Relationships between MV isolates of the D3 genotype in Spain (15.10.93–21.01.96). Numbers of expressed (circled) and silent (uncircled) mutations are shown.

Table 2. Nucleotide changes in the H genes of BJAB- and Vero-grown viruses

Virus	Position	BJAB-grown	Vero-grown	Effect
DL	1656	A	G	S → G at position 546
WTF	595	T	C	I → T at position 192
WTF	989	G	A	Silent
WTF	1656	A	G	S → G at position 546
WTF	1760	T	C	Silent
WTF	1856	T	C	Silent
WTF	1897	T	C	3′NCR (silent)

sequences reported by Hummel *et al.* (1994) have also been included in this analysis (Fig. 1). These appear to be extremely different from those of the original lineage group A Philadelphia-26 strain from which they are derived. There are 73 mutations in this case, 46 silent and 27 expressed. The *e/s* ratio was thus only 0·59, but the absolute number of mutations was extremely high as reflected in the position of these two sequences in the tree diagram (Fig. 1). Adaptation to rodent tissues, which do not express the primate CD46 MV receptor, may thus be associated with substantial mutations in the H gene and protein.

Salt-dependent haemagglutinin

Certain strains of MV only haemagglutinate in the presence of high molarities of salt (Shirodaria *et al.*, 1976). A salt-dependent haemagglutinating derivative of the Edmonston vaccine strain Edm P9 has been sequenced and compared to the parent vaccine (Alkhatib & Briedis, 1986). The only nucleotide change that has taken place in the Edm P9 strain sequence is that nucleotide 361 has changed from C to A giving rise to a

mutation in the H protein at position 117 (F → L). This was the only example of variation at this position. However, the recent isolates from Coventry (Outlaw & Pringle, 1996) showed that three had a mutation at position 360 (T → C) which also gives rise to the F to L amino acid change at position 117. The significance of this change is presently not clear and could be evaluated by the assessment of the salt dependency of the haemagglutination phenotype of the Coventry isolates.

Is there any evidence for recombination in MV?

Except for the different arrangements around the central nodes in the tree which are, as discussed above, not supported well in bootstrap analyses, a single potential case of recombination could be inferred from the tree shown in Fig. 1. The Boston 83 (Bo83) isolate was one of two strains representative of genotype G in the N gene tree (Rima *et al.*, 1995*a*); the other was an isolate made in the same year in Berkeley (California, USA). However, in the tree based on the H gene sequences (Fig. 1) it is more associated with group D which represents a number of North American isolates.

Closer analysis of the N gene sequences indicates that the Bo83 virus displays a number of U to C and A to G changes characteristic of those found in biased hypermutation of MV (Cattaneo *et al.*, 1988). These changes may be responsible for its very extreme position in the N gene tree and much more sequence information with the determination of a cross-over point would be required before it could be concluded that these data indicate that recombination has occurred. The data sets assembled by Rota *et al.* (1995) also confirm the similarity between trees drawn on the basis of N and H gene sequences and thus the likely absence of recombination between various MV strains. The geographical isolation of genotypes (Rima *et al.*, 1995*b*; Rota *et al.*, 1995) and the small window for co-infection necessary for recombination makes it unlikely to occur and its absence in the current data sets not difficult to understand.

Receptor down-regulation

Recently, various groups identified CD46 (Dörig *et al.*, 1993; Naniche *et al.*, 1993) and moesin (Dunster *et al.*, 1994) as molecules associated with the receptor for MV in primate cells. Both these molecules are down-regulated, i.e. their cell surface levels are reduced after infection with MV or by contact of MV-infected cells with potential target cells (Schneider-Schaulies *et al.*, 1996). We have already noted that the ability to induce down-regulation of CD46 differs from strain to strain (Schneider-Schaulies *et al.*, 1995). Although it has been demonstrated that the ability to cause down-regulation is solely a property of the H protein of MV (Schneider-Schaulies *et al.*, 1996), the proposed genetic clustering does not place strains with and without CD46 down-regulation potential in distinct genotypes.

In conclusion, the sequence of the H gene of MV is variable but the variation is not as high as in the C-terminal part of the N gene. The variation between MV strains in the H protein coding sequence is maximally about 5% at the nucleotide level and $< 3\%$ at the amino acid level, when the strains that have been adapted to various rodent host cells are left out of consideration.

This level of variation is similar to that observed in the human parainfluenza virus type 3 strains (which differed maximally by 4·8% at the nucleotide level; van Wyke Coelingh *et al.*, 1988) and is much below the level of variation observed between the HN genes of Newcastle disease virus strains which differed maximally by 16·1% at the nucleotide and 12·1% at the amino acid level (Sakaguchi *et al.*, 1989). The remarkably low mutation rates and the sequence analysis present no convincing evidence for antigenic drift in the MV H gene. Nevertheless, there is a significant potential for variation and the sudden emergence of mutated viruses, for example strain IFF, indicates that it may be necessary to monitor MV strain evolution in the future, especially in the context of global or local eradication programmes.

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