RNA virus evolution and the control of viral disease

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Dedicated to the memory of Prof. Ferran Calvet i Prats who died in Barcelona on the 16th of June, 1988.

Abbreviations

AIDS, acquired immune deficiency syndrome; DI, defective interfering; ds RNA, double-stranded RNA; FMDV, foot-and-mouth disease virus; HIV, human immunodeficiency virus; IV, influenza virus; M, matrix; MV, measles virus; PV, polio virus; RSV, respiratory syncytial virus; SSPE, subacute sclerosing panencephalitis; s/s/yr, substitutions per site per year; ts, temperature sensitive; VSV, vesicular stomatitis virus.

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1 Introduction: the dynamics of RNA genomes

RNA molecules display several features – some of which have been recognized only very recently – that contribute to their potential for modification and evolution. Cellular transcripts as well as autonomous RNA genetic elements may possess catalytic activity [1–3]. The following examples demonstrate the catalytic ability of RNA molecules, an activity which in the past was associated only with proteins. The active site of ribonuclease P – an enzyme that processes precursor tRNA molecules – resides in RNA [4,5]. Excision of the intervening sequence and subsequent splicing of *Tetrahymena* rRNA [6,7] is a protein-independent process. Regarding autonomous genetic elements, replication of viroids and certain plant virus satellites involves some protein-free RNA modification steps [8–12]. The catalytic potential of RNA includes hydrolysis of substrate RNA molecules *in trans* [13,14] and addition of nucleotides to preexisting chains [15–17]. Thus, biological catalysis, embodied in polynucleotide chains, adds to the potential for generating novel molecules in evolving RNA populations. This has strengthened the belief in a central role of RNA or of RNA-like molecules in the self-organization of a genetic memory and the early evolution of life on earth [18–20].

Present day RNA-replicating elements have developed other mechanisms of genetic variation to ensure their adaptability. Molecular recombination, initially thought to occur mainly in DNA, has now been shown to play an important role in several positive stranded RNA viruses [21]. [An RNA virus is positive stranded when the polarity of virion RNA coincides with that of mRNA; if virion RNA is of opposite polarity to mRNA the virus is negative stranded.]. Early observations on RNA recombination were made by selecting progeny picornaviruses from mixed infections with two parents harboring distinguishable selectable markers [22–24]. Direct proof of covalent linkage between two different parental molecules to yield a recombinant virus was obtained with foot-and-mouth disease virus (FMDV) [25] (review in [21]). RNA recombination occurs in animal and plant viruses [26] and in phage [27]. It may involve homologous, very closely related genomes, or very divergent molecules such as a cellular tRNA and viral RNA [28]. The molecular events leading to those different kinds of recombinant molecules are not known at present [21]. Poliovirus (PV) recombinants occur at high frequency *in vivo*. In a child fed all three

Sabin vaccine serotypes, the majority of novel antigenic variants shed during a 50-day period were type 2-type 3 intertypic recombinants [29]. In the coronavirus mouse hepatitis virus RNA, recombination is frequent in tissue culture [30] and in the brain [31], suggesting that it plays a role in natural evolution and pathogenesis of coronaviruses [31].

Recombination is distinguished from reassortment, exchanges of entire genome segments which occur among viruses with segmented genomes such as the influenza viruses (IV). New pathogenic influenza viruses have arisen by reassortment between human viruses and viruses from an animal reservoir (reviews in [32,33]). When it involves segments that encode the immunologically relevant surface antigens hemagglutinin or neuraminidase, the process is known as antigenic shift. Reassortments and RNA recombination events may occur simultaneously, as shown with rotaviruses, segmented double-stranded (ds) RNA viruses that cause diarrheal disease in infants. In chronically infected, immunodeficient children, rotaviruses with atypical genomic profiles were found; segment 11 was missing and additional ds RNA bands consisting of concatemers of segment-specific sequences were observed [34]. Rotaviruses with rearranged genome segments do reassort, and rearranged ds RNA can replace normal RNA segments functionally, and can also be replaced by normal RNA segments [35,36]. Reassortment and recombination permit large evolutionary jumps in RNA by bringing together genes or gene segments initially present in distinct ecological niches. Mutations that arise and are unfit in one genetic context can become viable or even advantageous in another context.

Probably the most widespread mechanism of RNA variation is mutation. Elements with RNA as genetic material (RNA viruses, retroviruses, viroids and satellites) or that utilize RNA as a replication intermediate (hepadnaviruses such as hepatitis B, retroposons, retrotransposons) mutate at rates estimated at about one million-fold higher than their host cells [37]. Because of their high mutability and their tolerance to accept change while remaining functional, RNA genomes are extremely heterogeneous collections of molecules (section 3). This structure for RNA viruses is relevant to the development of resistance to antiviral agents (section 6) and to difficulties encountered in the use of some anti-viral vaccines (section 7). Because of these and other implications for human and animal disease, current results on mutation rates and frequencies for RNA viruses, (section 2), as well as the mean-

ing of the extremely heterogeneous *(quasi-species)* nature of viral populations (section 4) are also reviewed. The subject has drawn increasing attention, as seen by the recent articles that have covered theoretical [37–43,49] as well as practical [37,41,44–48] implications of RNA genome evolution.

2 RNA versus DNA mutation rates, mutation frequencies and fixation of mutations

Mutation rate is the frequency of a misincorporation event during a single replicative round of nucleic acid synthesis. For the discussion that follows, it is important to distinguish *mutation rate* from *mutation (or mutant) frequency,* which is the proportion of genomes with a mutated residue in an RNA population. *Rate of fixation of mutations* is the number of mutations which per unit time become dominant among replicating genomes. *Fixation* may also refer to new dominant genomes during infections in cell culture (even during the development of a single plaque on a cell monolayer) or in host organisms, without a time factor being necessarily quoted. Some published values do not conform to the definitions given here, making comparisons of mutation rates and frequencies between viral systems difficult. This point is discussed in depth in ref. [49].

Several measurements suggest that mutation rates for cellular DNA are in the range of 10^{-7} to 10^{-11} substitutions per nucleotide per replication [50–53]. Mutations in DNA may arise by base pairing of rare tautomers of the usual bases, purine-purine mispairs with the free nucleotide substrate in the *syn* configuration, and by depurination of DNA, among other mechanisms [51–55]. The fidelity of copying is a result of the inherent accuracy of nucleotide incorporation and the subsequent proofreading step [56]. If this latter activity is suppressed, as in some mutant DNA polymerases, or by using homopolymeric templates, error levels of 10^{-3} to 10^{-4} per nucleotide are attained [51–53]. DNA hypermutability has been described in immunoglobulin gene segments [57–59], and in certain shuttle plasmid vectors during their replication in mammalian cells [60]. Transformed cells show increased genetic instability [61–63], that probably causes tumor cell heterogeneity [64,65], an important property for invasiveness and metastatic potential [61,65]. The basis of DNA genetic instability is not understood, with transposition of mobile elements and mutational events being prob-

ably involved. It has been proposed that transient DNA hypermutability may have contributed to accelerated evolution at certain times, thus determining punctuated rather than gradual evolution [66–68].

Rates of fixation of mutations in cellular genes (estimated by comparing homologous genes from organisms which diverged at times suggested by the fossil record) are in the range of 10^{-8} to 10^{-9} substitutions per nucleotide site per year (s/s/yr) [69–72]. Up to five-fold differences have been estimated among phylogenetic groups, the slowest values being for some bird lineages and primates (the so-called "hominoid slowdown" [70]) and the fastest for rodents, sea urchins and Drosophila [71]. These variations have been attributed to the different generation times among species or to the development of more efficient repair mechanisms [70,71,73].

Mutation rates and frequencies for RNA viruses have been estimated to be $10^{-3}$to $< 10^{-7}$ substitutions per nucleotide and genome doubling, using a variety of procedures (recent reviews in [44,49]). An extracistronic mutant (A (-40) → G) of phage Qβ prepared by site-directed mutagenesis [74] reverted at a rate of 10^{-3} to 10^{-4} per RNA doubling [75]. The transition G (-40) → A was quantitated by direct chemical analysis of the proportion of wild type and mutant sequence in the evolving population [74]. This was possible because the mutant sequence replicated less efficiently than its wild type counterpart [74]. Transversions G(-40) → C or G(-40) → U could possibly occur, but never be revealed in the analyses because they did not endow the genome with a measurable selective advantage over the mutant RNA. Because of the high mutation rates, populations of phage Qβ are genetically heterogeneous [76] (see section 3).

Steinhauer and Holland have developed a procedure to detect nucleotide sequences present at very low levels in RNA populations [77]. The method uses the absolute specificity of ribonuclease T1 for G residues. Selected RNA segments with a G residue flanked by sequences with only A, U and C are protected from ribonuclease (RNase) hydrolysis by hybridization with a complementary deoxyoligonucleotide. The protected RNA segment is then digested with RNase T1 and an "error oligonucleotide" is obtained when the molecules do not include the G residue. The presence of mutated bases is confirmed by sequencing. The proportion of "error oligonucleotide" relative to the two "consensus", shorter oligonucleotides permits a calculation of the mutation frequency at the G site [77]. A total of 57 clonal RNA preparations of

vesicular stomatitis virus (VSV) wild type and temperature sensitive *(ts)* mutants, with different passage histories have consistently provided error frequencies of 1 x 10^{-4} to 1 x 10^{-3} for G sites from the N, M and L genes and 5′-extracistronic region of the genome [78]. In a different experimental approach, the frequency of revertants of *amber* nonsense mutants of VSV was 10^{-3} to 10^{-4} [78a].

Measurements with other RNA viruses have yielded, however, lower error frequencies. The *ts* character of a Sindbis virus mutant reverted at a frequency < 5 x 10^{-7}[79]. It is not clear, however, that the phenotypic change was due to a single substitution. An *amber* mutant in a serine codon (position 28) in the poliovirus 3D (polymerase) gene reverted to wild type with a frequency of 2.5 x 10^{-6} [80]. The significance of this value is unclear because the selection of amber mutants involved small, slowly growing plaques, and early-arising wild type revertants could not have been scored as components of an amber plaque (small plaque) population. However, it is possible that this site has a low mutation rate.

Dougherty and Temin have constructed vectors that contain sequences from the avian retrovirus spleen necrosis virus plus several selectable markers [81]. Based on the expression of neomycin resistance in one of such vectors, it was estimated that the total mutation frequency (point mutations, additions and deletions) after a single round of virus replication was 5 x 10^{-3} [81]. In a subsequent study, the rate of a transition A → G (from an *amber* UAG codon to wild type UGG) was estimated at 2 x 10^{-5} per replication cycle [82]. It is not possible at present to exclude that other substitutions occurred at the same residue and were selected against [82]. The base insertion rate was about 10^{-7} per base pair per replication cycle [81].

Palese and colleagues have carried out repetitive sequencing of viral genes from clonal viral preparations derived from single plaques [83]. The rate of fixation of mutations was estimated in 1.5 x 10^{-5} for influenza NS gene and < 2.1 x 10^{-6} for polio VP1 segment [83]. In an extension of the same type of calculation, progeny from a single virion of Rous sarcoma virus was analyzed at several genomic sites by denaturing gradient gel electrophoresis [84]. The frequency of mutation was about 1 x 10^{-4} [84], a value that agrees with other estimates for Rous sarcoma virus [85].

The fidelity of RNA- and DNA-dependent RNA polymerases has been measured using enzyme preparations in cell-free systems. For

several retroviral reverse transcriptases, misincorporation frequencies ranged from 10^{-3} to 10^{-5} per nucleotide [86–90]. For PV RNA polymerase, Ward *et al.* [91] measured error frequencies of 7×10^{-4} to 5×10^{-3} using synthetic templates. All those measurements are influenced by the nature of the enzyme and of the templates, ionic composition of the reaction buffer, relative nucleoside-triphosphate concentrations, etc. [51,53], and it is not possible to assess at present to what extent they reflect error rates *in vivo*.

The limitations of several of the above measurements have been discussed by Eigen and Biebricher [49], who have compared values derived for DNA and RNA genomes. One difficulty is the evaluation of the relative fitness of the mutant molecules generated. Until many RNA sites for different RNA viruses, strains and isolates are analyzed using the same procedure, it will not be possible to reach conclusions on constancy or variation of mutation rates for different viruses or sites on a genome. It is even possible that a value for one site deviates from those at other sites, the latter being significant for one biological activity of the virus. For example, for Sindbis virus the lowest mutation rate so far calculated for an RNA virus ($< 5 \times 10^{-7}$, ref. [79]) was reported. Yet, antigenic variants were found at frequencies of $10^{-3.5}$ to 10^{-5} [92], not far from values for other viruses [93].

There is no basis to support the notion that the replication of some groups of RNA viruses (including retroviruses and HIV) is more error-prone than the replication of others. In keeping with theoretical concepts of Eigen and colleagues [43,49], RNA viruses may derive a selective advantage by maintaining their replication fidelity near the "error threshold" for stable information (section 8). In this situation, mutation rates would not differ by more than ten-fold [49,93,94], perhaps with the bias towards higher fidelity for larger, unsegmented genomes because of their increased information content within a single genomic molecule [43,49].

The rates of fixation of mutations during propagation of RNA viruses in nature may reach exceedingly high values nearing 10^{-2} s/s/yr. An interesting comparative figure is the 10^{6}-fold higher rate for the viral v-*mos* gene relative to its cellular counterpart c-*mos* [95]. The NS gene of influenza A virus showed a uniform rate of evolution of 2×10^{-3} s/s/yr [96], a value similar to that of the neuraminidase N2 gene [97]. Rates for the *env* and *gag* genes of the AIDS virus were 1×10^{-2} to 3×10^{-3} and 1.85×10^{-3} to 3.7×10^{-4} s/s/yr [98,99]. Gebauer *et al.* [100] sequenced the

VP1 gene of foot-and-mouth disease virus (FMDV) during an inapparent, persistent infection of cattle that was established with plaque-purified virus [101]. They measured an evolution rate of 9 x 10^{-3} to 7.4 x 10^{-2} s/s/yr in a single animal [100], a range similar to that estimated during an episode of acute disease [102], or for lentiviruses [102a]. In contrast, a long-term conservation of the VP1 coding region of FMDV was evidenced by sequence comparison of two viruses isolated over a 29-year interval [103]. An extreme case of long-term conservation has been observed by Gibbs and colleagues [42] with a strain of turnip yellow mosaic tymovirus that suggest at most 1 % nucleotide variation during the past 12000–15000 years. That the same virus shows a dual potential for rapid variation and for long-term conservation has been clearly demonstrated by Holland and his colleagues using VSV [37,45,104–110]. Rapid evolution was driven by defective-interfering (D.I.) particles during high multiplicity passages or in the course of persistence in cell culture [104–109]. In the absence of selective pressures, the same or very similar average sequence can be maintained for many generations [110] (see also section 4).
Again, from the data presently available no bias towards higher rates of fixation of mutations or increased potential for long-term conservation of sequences for any group of virus is apparent.

3 Genetic heterogeneity of RNA viruses

A considerable wealth of evidence indicates that populations of RNA viruses are genetically heterogeneous. By fingerprinting RNA from individual clones of phage Qβ [76] it was estimated that, assuming a random distribution of the mutations found, each infectious Qβ genome differs in one to two positions form the "average" or "consensus" sequence (see section 4). Many field isolates and laboratory-adapted populations of RNA viruses have proven genetically heterogeneous. This is also true for clonal pools, derived from a single infectious unit. Multiple variants are present in a single infected organism, as shown for FMDV [111,112], subacute sclerosing panencephalitis (SSPE) virus 113–115] and more recently for IV [116] and the AIDS viruses [117,118], among others. Independent isolates of the same virus are, in general, genetically distinct (recent review in [44] and Table 1 for selected examples).

Table 1
Heterogeneity of RNA genome populations

Virus	Type of population	References
Phage Qβ	Clonal populations	76
Vesicular stomatitis virus	Field isolates	119–121
Measles virus	Clinical isolates	122
Subacute sclerosing panencephalitis virus	Among viral molecules of an infected brain	113–115
Avian paramyxoviruses	Field isolates	123, 124
Lymphocytic choriomeningitis virus	Comparison of strains	125, 126
Bunyaviruses	Comparison of strains	127, 128
Influenza virus	Clonal populations	83, 129
	Infected human	116
Poliovirus	Clinical isolates	29, 130–135
	Clonal population *in vivo*	135a
Foot-and-mouth disease virus	Field isolates	136
	Clonal populations	137, 138
	Clonal populations *in vivo*	100
Enterovirus 70	Clinical isolates	139
Coxsackievirus A10	Clinical isolates	140
Drosophila C	Natural isolates	141
Hepatitis A	Clinical isolates	142
Venezuelan equine encephalomyelitis	Field isolates	143
Western equine encephalomyelitis	Field isolates	144
Sindbis	Field isolates	145
St. Louis encephalitis	Clinical isolates	146
Dengue	Clinical isolates	147
Yellow fever	Clinical isolates	148
Murray Valley encephalitis	Clinical isolates	149
Murine hepatitis	Comparison of strains	150, 151
Tobacco mosaic virus	Comparison of stocks and strains	152, 153
Reovirus	Natural isolates	154, 155
Rous sarcoma virus	Natural isolate	156
AIDS viruses	Clinical isolates	117, 118
Hepatitis B	Chronic carrier	157
Yeast killer elements	One isolate	158
Viroids	Natural isolates	159–162a

Figure 1 depicts homogeneous and extremely heterogeneous genome populations. Lines represent genomic molecules, sprinkled with mutations in populations B and C, but not in A. It is noteworthy that the sets A and B share the same "consensus" or "average" sequence (the one that includes in each position the residue most represented in the set of molecules). However, A and B differ in the nature of the individual genomes that comprise the population. Since individual molecules cannot be sequenced without prior amplification, the heterogeneous nature of population B can only be revealed after biological or molecular cloning (arrow in Fig. 1). A new distribution C with a "consensus" or

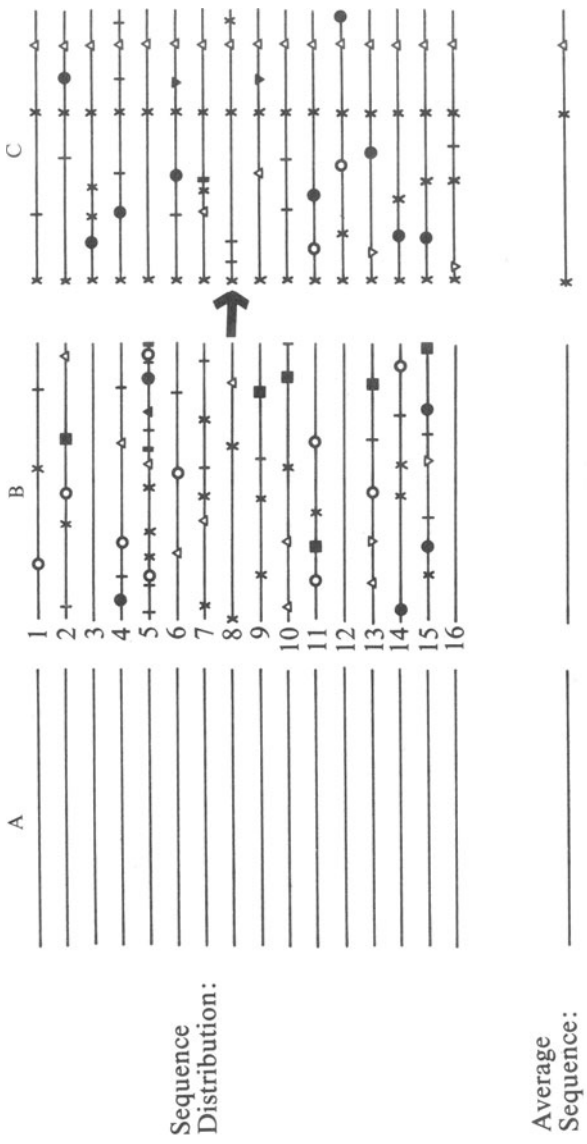


Figure 1
Diagram of homogeneous (A) and heterogeneous (B and C) genome populations. Each line is an RNA molecule, and symbols on the lines are mutations. The average sequence contains in each position the most frequent nucleotide in the sequence distribution. Note that in spite of the heterogeneity in B (average of 4 mutations per genome) this distribution has the same average than A. The arrow represents the amplification of molecule 8 from distribution B to yield a new distribution C, with a new average. Events such as plaque purification of a virus on a cell monolayer or transmission of a single infectious virion from one infected to a susceptible host are equivalent to the generation of C.

"average" sequence different from B is generated. Molecules 3, 12 and 16 in set B coincide with the "average", but they are a minority in the population. Sampling of nucleotide sequences has indicated that RNA viruses consist of pools of variants as in sets B or C. The shape of the distributions (average number of mutations per genome, standard deviations from the mean, etc.) are largely unknown, but the available evidence suggests that those parameters vary for different viruses and different populations.

4 Population equilibrium and quasi-species

There are two remarkable features of RNA genome distributions not reflected in Figure 1: (i) the extremely large population size, and (ii) the variations in the composition of the nucleotide sequence distribution with time. Measurements of the number of infectious viral particles in several organisms have yielded 10^9 to 10^{12} per infected individual (reviewed in [41]). Given the size of RNA viral genomes (3000 to 30000 residues), taking a range of heterogeneity for clonal pools of one to ten mutations per genome (several experimental measurements are included in Table 1) it can be calculated that all possible single and double mutants, as well as decreasing proportions of triple, quadruple, etc. mutants, are *potentially* present in most natural viral populations [41,76,93]. The proportion of a variant in an evolving population will depend on the rate at which it arises as well as on its fitness relative to the other variants (present or arising) in the population. The studies of Weissmann and colleagues with phage $Q\beta$ [74–76] led to the proposal that "a $Q\beta$ phage population is in a dynamic equilibrium with viable mutants arising at a high rate on the one hand, and being strongly selected against on the other. The genome of $Q\beta$ phage cannot be described as a defined unique structure, but rather as a weighted average of a large number of different individual sequences" [76]. At least some aspects of such a description apply to animal and plant RNA viruses as well ([37,41,44,110] and Table 1). This has lead to the proposal of the "population equilibrium model" for RNA genomes [93]. Little is known on the mechanisms that maintain equilibrium: What is the proportion of neutral or quasi-neutral variants relative to disadvantaged variants? What are the chances of generating advantageous combinations of mutations in constant or changing environments? Several such questions are now under investigation. That a relatively stable equilib-

rium can be maintained for many generations (leading to long-term conservation of nucleotide consensus sequences) in spite of considerable genetic variation among the individual molecules that constitute that population, has been shown by Holland and his colleagues using VSV [110]. Under low multiplicity passage, the consensus T1 fingerprint of VSV RNA remained unchanged after 523 passages. Yet, RNA from individual clones from this population differed from each other and from the consensus in an estimated 10 to 40 positions [110]. Thus, there is no incompatibility between long-term conservation of sequences and an extremely heterogeneous viral population.

The conclusions of the experimental analyses summarized above, are highly consistent with those predicted by the *quasi-species* concept, proposed by Eigen and colleagues to describe the error-prone replication of molecules during the early evolution of life on earth [39,43,49,163,164]. Such molecules will be organized in distributions dominated by one (or several) *master sequence(s)* and a *mutant spectrum*. The *master* may, nevertheless, represent a small proportion of the total population. As an example, molecules 3, 12 and 16 in set B of Figure 1 can be regarded as the *master sequence;* it coincides with the consensus and represents 18 % of the total. To what extent the theoretical studies on *quasi-species* will help in understanding the behavior of RNA viruses is currently the object of considerable research. Fontana and Schuster have used computer modeling to study the dynamics of folding binary sequences into unknotted two-dimensional structures [165]. This simulation showed features typical of the evolution of real populations. Displacement of one sequence distribution by another, each centered around a different *master* was observed [165].

Biebricher *et al.* have studied replication of small, variant RNA molecules by $Q\beta$ replicase [39,166,167]. The RNAs were highly heterogeneous in sequence, rendering direct RNA sequencing difficult [166]. They showed that mutant frequencies are not determined by mutation rates alone, since selection forces play a dominant role [166,167]. This experimental system may be suitable to test predictions of the *quasi-species* model, prior to designing experiments with complex RNA genomes.

In addition to its inherent interest, the *quasi-species concept* has been very timely in providing a strong theoretical background to the studies of RNA virus evolution. Despite the concept having originated in a seemingly unrelated discipline, the relevance of *quasi-species* for

RNA viruses is strongly supported by the available evidence [47–49,76–78,83,84,93,110–118,129]. It certainly has helped in regarding virus populations as "organized combinations of macromolecular species" [163,164] rather than defined nucleotide sequences.

5 Phenotypic heterogeneity

Single nucleotide or amino acid replacements may greatly affect the behavior of macromolecules, including RNA viruses [41,74,93]. Viruses selected for resistance to one monoclonal antibody (MAb) often show one amino acid change at the relevant epitope ([93] and references therein). A substitution in the hemagglutinin H3 changed the receptor binding specificity of IV [168,169]. Similarly, a single conservative amino acid change on human immunodeficiency virus (HIV) gp 120 drastically reduced its recognition by T cell clones [170]. Two or a few mutations may affect virulence, host-range or particle stability [41,93]. Thus, viruses with altered phenotypes are present in the "mutant spectrum" of the genome distributions (Fig. 1, and sections 3 and 4). "It is as if an RNA virus encompasses a *range of phenotypes* able to express themselves with a certain probability in a given environment" [93]. Let us examine the development of resistance to antiviral drugs, considering the population equilibrium structure of RNA viruses.

6 Resistance of RNA viruses to antiviral agents

If a single mutation is able to confer resistance to an antiviral agent, and the mutation does not cause a significant selective disadvantage to the virus in the considered environment, a drug-resistant virus mutant will be present in most, if not all, virus populations. The expected proportion according to recent direct measurements of mutated residues in RNA populations is up to 10^{-3} to 10^{-4} [49,77,78]. The actual value for each site depends on the mutation rate and fitness of the variant molecule (section 4). The likelihood of finding a resistant virus will decrease with the number of mutations required to provide the resistance phenotype; not only because of the lower probability of generating it, but also because of increased chances of it being less fit. If any possible mutation able to confer resistance diminishes viral fitness substantially, resistance will not develop under those environmental conditions. Of course, failure to derive resistant mutants may also signify

either that a large number of mutations – above the range expected to be represented in a *quasi-species* – are required or, more trivial, that the drug acts in a rather unspecific manner [171].
Antiviral agents may interact with virions and prevent their infectivity by inhibiting an early step in infection such as attachment to the host cell, penetration or uncoating. Others interfere with the intracellular viral nucleic acid or protein syntheses, protein processing, virion assembly or release from cells. The mechanism of action of a wide range of antiviral agents and the problem of development of resistance have been reviewed recently [172,173]. Here we will discuss selected antivirals for which some quantitation of detection of resistant variants has been made and the molecular mechanisms of drug action are understood at least to some extent.

6.1 Drugs that act at an early stage of the virus infectious cycle

Amantadine (1-aminoadamantane) (Fig. 2) and rimantadine (α-methyl-1-adamantane methylamine) (Fig. 3) are used for the prophylaxis and treatment of IV type A infections [174–177]. Resistant mutants have been obtained in tissue culture upon passage of the virus in the presence of the drug [178–180], in animals [181,182] and from humans subject to treatment [185–187]. The isolated variants tested showed cross-resistance to the two drugs, suggesting that both act by the same or a very similar mechanism. Resistance maps in the matrix (M) gene [180,187–189], although a possible influence of other gene products has not been excluded [187,190,191]. The surface antigen hemagglutinin may be indirectly involved as a result of its interaction with protein M2 [191]. Different human IV A strains vary in their sensitivity to these drugs [179,187]. For most human IV strains, an early stage in the infection is inhibited. For avian viruses, however, the late assembly step appears to be the target. Perhaps critical interactions between M2 and hemagglutinin that occur both during penetration and assembly are affected to a different extent in the two steps, depending on the viral strain [190]. Amantadine-resistant mutants of IV A show single amino acid substitutions in M2 [188,191]. Rimantadine resistance was associated with the corresponding genome segment 7 in reassortant viruses generated with a resistant clinical isolate [190]. Nucleotide sequencing showed the single amino acid changes Ala/30→Val or Ser/31 →Asn of M2 to be associated with the resistant phenotype [190]. Since the

Figure 2
Amantadine (1-aminoadamantane).

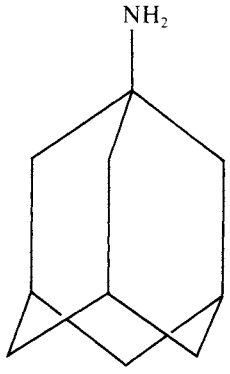
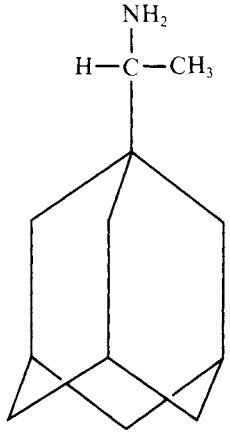


Figure 3
Rimantadine (α-methyl-1-adamantane methylamine).



variants replicated efficiently at least in cell culture, the results suggest that amantadine- and rimantadine-resistant mutants should occur frequently in IV populations. Indeed, their proportion in various IV preparations was 1 x 10^{-3} to 4 x 10^{-4} [179,180]. Among children treated with rimantadine, 27 % shed resistant viruses [190]. Thus, selection of resistant IV variants is likely to be a frequent event, and an important problem mainly during therapeutic use of these drugs, since selection is then exerted on a large pool of replicating genomes.

A number of drugs bind to the capsid of picornaviruses and inhibit their uncoating. Dichloroflavan (4′, 6-dichloroflavan) (DCF) (Fig. 4), 4′-ethoxy-2′-hydroxy-4, 6′ dimethochalcone (RO-09-0410) (Fig. 5), 2-[-(1,5,10,10a-tetrahydro-3H-thiazolo [3,4b] isoquinolin-3-ylindene) amino]-4-thiazole acetic acid (44-081 R.P.) (Fig. 6), disoxaril, 5-[7-[4-(4,5 dihydro-2-oxazolyl) phenoxy] heptyl]-3-methyl-isoxazole (WIN

51711) and its methyl derivative WIN 52084 (Fig. 7) and arildone (4-[6-(2-chloro-4-methoxyphenoxy) hexyl]-3, 5-heptanedione (Fig. 8) belong to this group. Crystallographic analysis has shown that WIN 51711 and WIN 52084 go to the hydrophobic interior of the VP1 β-bar-

Figure 4
Dichloroflavan (4′,6-dichloroflavan).

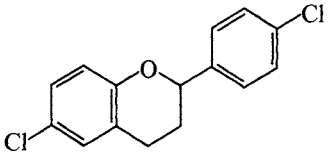


Figure 5
4′-ethoxy-2′-hydroxy-4,6′ dimethochalcone (R0-09-0410).

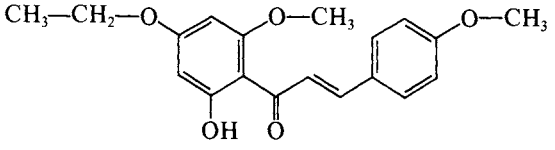


Figure 6
2-[-(1,5,10,10a-tetrahydro-3H-thiazolo[3,4b]isoquinolin-3-ylindene) amino]-4-thiazole acetic acid (44-081 R.P.).

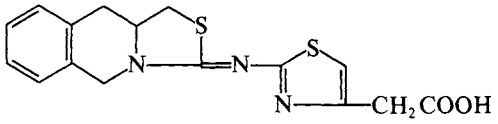


Figure 7
R = H: Disoxaril, 5-[7-[4-(4,5 dihydro-2-oxazolyl) phenoxy] heptyl]-3-methyl-isoxazole (WIN 51711). R = CH_3, WIN 52084.

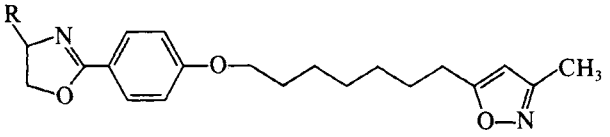
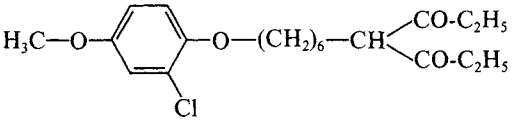


Figure 8
Arildone (4-[6-(2-chloro-4-methoxyphenoxy) hexyl]-3,5-heptanedione.



rel of human rhinovirus 14 [192]. Much emphasis has been put on the conservation of the general architecture of picornaviruses [193]. Since these drugs accommodate into a pore of the viral capsid, one would anticipate that most viral strains would be equally sensitive, and that resistant mutants would be rare. However, the antiviral activity of these agents varies up to 10^3-fold for different rhinovirus serotypes [194–197]. Moreover, low level resistance to disoxaril was found at frequencies of 10^{-3} to 10^{-4} and high level resistance at 10^{-5} (quoted in [197]). A rhinovirus 9 mutant resistant to the chalcone R0-09-0410 was found at a frequency of 10^{-5}, and grew less efficiently than wild type [198]. It is not known whether resistant strains would generally show a reduced fitness. Clinical trials have yielded poorer results than anticipated from cell culture assays due in part to insufficient drug concentration in the target tissues. This increases the chances of selecting variant viruses with low-level resistance *in vivo* [199].

There are more than a hundred serotypes of rhinovirus, suggesting an increased difficulty in finding effective, wide-spectrum anti-rhinovirus agents. However, 90 % of the serotypes interact with the same cell receptor [200]. A MAb directed to such receptor, effectively competed with virus binding [201], but prevention of infection or illness *in vivo* was not observed [202]. Interference with virus attachment and penetration has also been achieved with synthetic peptides that represent amino acid sequences from the viral surface needed either for receptor recognition or membrane fusion [203,204]. Choppin and colleagues sequenced the fusion (F) protein gene of a measles virus mutant resistant to fusion inhibiting polypeptides [205] and identified three amino acid changes located at the carboxy terminal half of F. It is not possible to know at what frequency the mutant arose since it was selected by repeated passage of single plaques in the presence of one oligopeptide (quoted in [205]). An alternative approach is to inhibit virus attachment by soluble receptor protein, as currently tested with CD4 to prevent HIV infectivity [206]. Many clinical trials using peptides and soluble receptor proteins are currently in progress with AIDS patients [207]. Little is known of the possibility of selecting variant viruses with altered receptor specificity, as shown previously for Coxsackie virus in cell culture [208]. Most viral receptors probably play a role in normal cell physiology [208a], and it is currently a concern that their blockade or manipulation may have undesirable side effects.

6.2 Drugs that act on an intracellular step of virus development

Some nucleoside analogs are very effective antiviral agents. Usually they are activated with the participation of virus-coded enzymes, and inhibit a viral nucleic acid polymerase. A classical antiherpes drug, acyclovir or 9-(2-hydroxy-ethoxymethyl) guanine, is converted into the monophosphate form by the viral thymidine kinase and then into the active triphosphate by cellular enzymes. The molecular basis and clinical importance of resistance to acyclovir have been recently reviewed [209]. A number of analogs inhibit the multiplication of RNA viruses. Ribavirin, (1-β-D ribofuranosyl) 1-*H*-1,2,4-triazole-3-carboxyamide) (Fig. 9) is a broad spectrum antiviral agent [210–212]. Ribavirin lowers the intracellular pool of GTP by inhibiting inosine monophosphate dehydrogenase [213], resulting in inhibition of viral polymerases, or capping of mRNA [214], or both. Many viruses that cause important diseases are effectively inhibited by ribavirin. For FMDV, the drug was ten-fold more effective in inhibiting viral replication during a persistent than during a lytic infection [215]. It has proven particularly successful when applied as an aerosol at the upper respiratory tract, for influenza and respiratory syncitial virus infections [216], and in some severe infections such as Lassa fever and Argentinian hemorrhagic fevers [217,218]. It has been difficult to derive ribavirin-resistant mutants [219]. The only example, to my knowledge, is a Sindbis virus (SV) mutant derived by Scheidel *et al.* [220]. By serial passage of SV in the presence of inhibitory concentrations of mycophenolic acid, mutants resistant to the drug, that showed cross-resistance to ribavirin, were obtained. They suggested that an altered viral enzyme – RNA polymerase or, more likely, RNA guanylyltransferase – was selected that

Figure 9
Ribavirin, (1-β-D ribofuranosyl) 1-H-1,2,4-triazole-3-carboxamide.

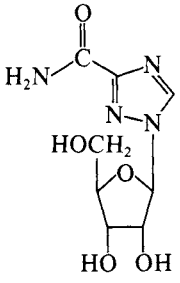
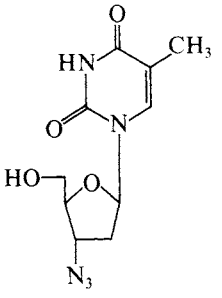


Figure 10
3′-azido-3′-deoxythymidine (AZT or retrovir).



was functional at lower GTP concentrations than those required by the wild type enzyme [220].
The 2′, 3′-dideoxynucleoside analogues are inhibitors of retrovirus replication [221]. They are converted into the 5′-triphosphate form by cellular kinases, and act as chain terminators during reverse transcription [222]. The analog 3′-azido-3′-deoxythymidine (AZT or retrovir) (Fig. 10) is currently being tested in AIDS patients, with promising results ([207,221] and references therein). Recently, AZT-resistant HIV variants have been isolated from AIDS patients [222a]. Selection of such mutants may be quite widespread, in view of the lengthy treatments required for this disease.

Many other nucleoside analogs are currently under investigation, and because they often affect the active sites of essential viral enzymes, it may be more difficult to select resistant variants and may prove active against a wider range of naturally occurring variants. It is encouraging that no differences were noted in the inhibitory activity of 20 nucleoside analogues (including ribavirin and 3-deazaguanine) on ten strains of IV types A and B and one isolate of type C [223].
Guanidine (Fig. 11) inhibits several animal and plant viruses, including picornaviruses. Its action on FMDV and PV is at the RNA synthe-

Figure 11
Guanidine.

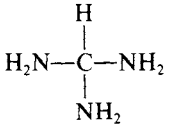
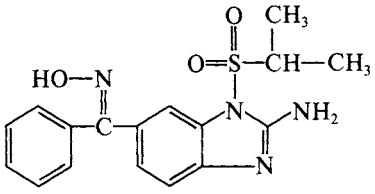


Figure 12
Enviroxime, anti-6-[(hydroxyimino)-phenyl-methyl]-1-[(-methylethyl) sulfonylimidazol-2-amine].



sis step, and after some conflicting results, there is now good evidence that mutations that confer guanidine resistance or dependence map in viral polypeptide 2C (or P34) [224,225]. PV type 1 mutants resistant to 2mM guanidine (there are several degrees of resistance to guanidine) showed one amino acid substitution, Asn/179 → Gly or Asn/179 → Ala in 2C, that involved two nucleotide substitutions in each case [226]. Two guanidine-dependent mutants, selected upon serial passage of PV in 2 mM guanidine, had two amino acid substitutions each: Asn/179 → Gly (the same observed in resistant mutants), Ile/227 → Met in one mutant, and Ile/142 → Val, Met/187 → Leu in another mutant [226]. The mutation frequencies of PV to guanidine resistance were 1.8×10^{-5} to 4×10^{-8} [226]. These values are compatible with multiple mutations causing the resistance phenotype, as indeed observed by genomic sequencing. Infectious cDNA clones that included the relevant mutations, yielded the expected phenotypes, thus indicating the direct involvement of 2C [227]. The role of 2C in the picornavirus replication complex is not known, and thus the molecular basis of guanidine action remains undefined [228].

Enviroxime, (anti-6-[(hydroxyimino)-phenyl-methyl]-1-[(-methylethyl) sulfonylimidazol-2-amine]) (Fig. 12) is effective in inhibiting replication of many rhinovirus serotypes [229]. Recent evidence suggests that the drug may inhibit the formation of the replication complex (quoted in ref. [197]) and no resistant mutants have been studied [219].

6.3 Additional antiviral strategies

Many compounds, in addition to those discussed in previous paragraphs, are presently under study as antiviral agents [172,173,230]. Among them, interferons, now available in large amounts via recombi-

nant DNA expression systems, have generally given poor results in clinical trials [230], and more work is needed to understand the basis of their antiviral activity.

The expression of polynucleotides or viral polypeptides has induced antiviral activity in plant and animal cells. Short oligodeoxynucleotides or RNAs complementary to viral messengers ("antisense" RNA) have, in several instances, blocked virus replication (reviewed in [230]). Gene transfer techniques may allow the constitutive expression of such antiviral macromolecules in plants or animals. In addition to the possible selection of viral variants (a largely unexplored subject), it is uncertain whether living cells will maintain a stable expression of abnormal nucleic acids and/or proteins. The potential for cell variation is, at least on occasions, remarkable. The induction of multidrug resistance in cultured cells or in tumors is a pertinent example. Strategies for its reversal is key to antitumor therapy and an active field of research on its own (see also section 2).

6.4 Combination therapy

The early [171,231] and more recent studies on virus inhibitors summarized above (sections 6.1 to 6.3) demonstrate that the emergence and selection of virus variants resistant to antiviral agents is quantitatively significant, and a potential severe problem in medical practice. Frequent appearance of drug-resistant viruses is the expected consequence of the successive population equilibrium steps through which RNA viruses evolve (section 4). Even a very rare (infrequent) genome from the *mutant spectrum* of the *quasi-species*, when able to grow in the presence of an inhibitor of the replication of other members of the distribution, will be selected to form a new *quasi-species*. The process can be represented as the transition from distribution B to C depicted in Figure 1 (section 3). In this process, a new genomic distribution (not a single individual) will be selected that may adjust to be quite fit while maintaining the drug resistance trait, in the absence of the drug. Even wide-spectrum agents that profoundly affect biochemical parameters in the cell (example, depletion of the GTP pool by ribavirin) have proven capable of selecting resistant viruses, as illustrated by the isolation of a ribavirin-resistant mutant of SV [220] (section 6.2). We are dealing with inherently variable and indeterminate distributions of genomes, with ever-evolving mutant profiles (section 4). Such profiles are pres-

ently inaccessible to detailed analysis (except for computer simulations [165] and experiments with simple RNA molecules [166,167], section 4) since they would require the sequencing of thousands of viral genome molecules from several populations, a task that necessitates waiting for technical improvements. This indeterminacy of the profile of the distributions generates an uncertainty as to the proportion of drug-resistant mutants likely to arise in an evolving virus population. For viruses that can be grown in cell or tissue cultures, parallel serial passages in the presence of the drug should provide an experimental system to determine in a statistically reliable fashion the frequency of emergence of variants. It must be noted, however, that the results may not be relevant to viral multiplication *in vivo,* where the virus finds a different environment.

In light of the above considerations, the most adequate strategy for an antiviral therapy would be a *combination therapy* applied in cases of fatal illness or, exceptionally, a *combination prophylactic treatment* directed to groups at high risk of fatal illness. Indiscriminate, widespread use of antiviral agents should be avoided. Combination therapy has been discussed in several recent reviews [207,232,233]. It should involve non-antagonic, multiple drugs with an independent target site of their inhibitory action, or drug-interferon combinations [234] with synergistic activity. Synergisms and antagonisms are difficult to anticipate, as shown by the finding that ribavirin antagonizes AZT in its anti-HIV activity, apparently by inhibiting its phosphorylation [207]. Statistical considerations suggest that with an adequate combination therapy, the likelihood of selecting a variant with the multiple mutations required for multidrug resistance is many orders of magnitude lower than that of selecting for resistance to one drug. This point is testable in serial passage experiments in which the frequency of appearance of resistant viruses to one or several drugs can be compared.

7 Vaccines

The success of vaccination in controlling important viral diseases such as smallpox, poliomyelitis, mumps or measles [235–239] does not justify overlooking the problems encountered with current vaccines. Furthermore, for diseases of increasing concern, such as AIDS, vaccines are not available. The types of vaccines in use or under experimenta-

Table 2
Types of antiviral vaccines (from refs. [230, 238, 240, 241])

Inactivated, whole virus.
Live-attentuated (various mutants selected *in vivo* or produced by site-directed mutagenesis on cDNA copies of viral genomes; vaccinia or other recombinant viruses).
Synthetic (capsid proteins or structures; non-structural proteins [241]; oligopeptides).
Anti-idiotypic [240].

tion are shown in Table 2. Anti-idiotypic antibodies [240] provide reagents that may maintain the antigenic properties of complex protein conformations important in the immune response, and difficult to mimic with synthetic proteins or oligopeptides. Attenuated viruses such as vaccinia have been manipulated to include foreign viral antigens and considerable effort has been put in the development of such recombinant vaccines [230,242–244], not always successfully [245]. The chances of a vaccine inducing a protective immune response are higher when the response resembles that produced during a natural infection with the virus [238]. Obviously, a vaccine must be safe, stable, and available to a substantial proportion of individuals from the populations at risk [238].

During their replication, attenuated viruses undergo genetic variation (sections 2 to 4). PV serotype 2 and 3 vaccine strains can fix more than 100 mutations during replication in one or two individual humans [130]. A single nucleotide substitution at position 472 (from U in type 2 Sabin vaccine to C in mutants) occurred regularly during viral multiplication in the intestine of vaccinated persons [246]. This well-documented variability of PV (see also Table 1, section 3) has caused reversion of attenuated viruses to virulent forms and a number of cases of vaccine-associated poliomyelitis [247,248]. Among vaccinees and their contacts, the incidence of paralysis in different countries ranged from 0.13 to 2.29 cases per million doses of vaccine distributed [248]. Even in well-vaccinated communities, outbreaks of poliomyelitis have occurred among the unvaccinated individuals, as in China in 1982 [249]. The PVs isolated during an outbreak in Finland in 1984 were heterogeneous [135] and antigenically different from the previous isolates of PV types 1,2 or 3 [250]. The availability of infectious cDNA copies of the PV genome may permit the preparation of attenuated variants with low frequencies of reversion to virulence. However, this is proving a difficult task since the molecular basis of attenuation remains elusive. Recombinational analysis [251] and sequencing of PV variants [252]

has indicated that several loci spread over the vaccine virus genomes may influence the attenuation phenotype. It has been suggested that infectious cDNA clones of poliovirus Sabin strains may be used as a repository of inocula for vaccine production [253], reducing the risk of reversion to virulence by repeated passage. Antigenic chimaeras of poliovirus have been suggested as potential new vaccines [254]. However, the progeny from a cDNA clone will also be a *quasi-species* distribution of genomes, with a "range" of phenotypes (sections 5,6). Thus, eradication of poliomyelitis [255], as inevitable as it may seem, will probably still require considerable research effort.

Reversion from attenuated to virulent forms occurred with *ts* and cold-adapted *(ca)* mutants of IV [230,256] and *ts* mutants of respiratory syncytial virus (RSV) [256]. Some vaccinees shed *ts* + RSV revertants [256]. The attenuated RSV were assayed since an inactivated vaccine not only did not induce protection, but aggravated the clinical response of children to subsequent RSV infection [257]. Encephalitis following measles, mumps or rubella vaccination occurs at a low rate of about one case per million doses of vaccine [239], and the few cases of SSPE in children without previous evidence of measles, may also have a vaccine origin [258]. These observations emphasize a likely role of variant viruses in chronic, degenerative disease [37]. Attenuated viruses share with their wild type counterparts the potential for initiating atypical infections (see also sections 8,9) and for triggering immunopathological responses. In 1976–1977, many cases of Guillain-Barré syndrome in the U.S. were attributed to vaccination with influenza virus A/New Jersey [259], but the mechanisms involved are not clear.

7.1 Virus variation and vaccine design

RNA virus variation in the field is increasingly recognized as one of the main obstacles for vaccine efficacy [37,41,47,93,136,260–263] but, curiously, this problem was very often overlooked in the initial design of synthetic vaccines. Most human and animal viruses exist as several serotypes, subtypes and antigenic variants. Even for viruses considered antigenically stable such as hepatitis A, RSV or rabies, use of MAbs has shown antigenic differences between isolates [93,230]. Whether these differences may cause vaccine failures or not depends on a complex set of parameters, some of which are discussed below. A useful model system for vaccine studies is provided by FMDV, an

economically important pathogen that infects several animal species suitable for experimentation (mice, guinea pigs, swine, etc.). Seven serotypes and more than 65 subtypes of this virus were recognized by application of classical serological techniques (complement fixation and neutralization assays). A recent quantitation of the reactivity of field isolates of serotype C with MAbs directed at epitopes involved in neutralization of infectivity indicated an extensive antigenic heterogeneity, even among viruses of one disease outbreak [261,263]. Mateu *et al.* suggested that given the *quasi-species* nature of FMDV populations, each viral isolate may be not only genetically unique, but also antigenically unique when a large enough panel of MAbs is used to quantitate antigenic reactivities. Whole-virus inactivated vaccines prepared with each one of two viruses from the same FMD episode were able to protect swine against challenge with homologous virus, but only partially against heterologous virus [262]. In this case, amino acid substitutions fixed during the natural evolution of FMDV caused a discernible effect in vaccine potency. The same problem has recently become apparent when the genetic and antigen heterogeneity of the AIDS viruses has been recognized [98,99,117,118,264]. The conclusions on antigenic heterogeneity of FMDV and HIV are very similar to those reached previously by Prabhakar, Notkins and colleagues, comparing clinical isolates of Coxsackie virus B4 [265–267] and they may be common to many more pathogenic RNA viruses.

The spectrum of antigenic variants that at any one time will be represented in a viral population is indeterminate. For each epitope, there will be tolerance for certain amino acid substitutions. Some replacements will alter epitope reactivity. Not only variations fixed on the surface proteins, but also those in non-structural proteins may be immunologically relevant, since they may affect T-cell recognition of infected cells. The total number of epitopes involved in triggering the humoral and cellular responses leading to protection will influence the weight that changes in individual epitopes have in the ability of a virus to escape from an immune response directed to a related variant. If the equilibrium populations (section 4) remain stable, changes in consensus antigenic structure are unlikely and a vaccine may show long-lasting efficacy. A stable equilibrium may be maintained for many generations in spite of a remarkable genetic heterogeneity and high mutation rates (several examples in section 4). A fictional story might clarify the point. Assume phage Qβ had the nasty property of occasionally infect-

ing humans causing an abortive but fatal infection, from which no viable particles can be rescued. Imagine that such a fatal disease would be amenable to control by an inactivated Qβ vaccine, with phage produced in *E. coli*, the sole productive host of Qβ. Such a vaccine would maintain its efficacy from the times of Sol Spiegelman (see refs. [76,93] for an account of the passage history of Qβ), in spite of the undisputed high mutability and heterogeneity of Qβ populations (section 3). Thus, factors that favor genetic disequilibrium in natural populations of viruses will increase the likelihood of antigenic drift. Again, disequilibrium may be caused by environmental changes and transmission bottlenecks (or founder effect), in which one genome is greatly amplified [41,111]. The ability of many viruses to infect different host organisms and different cells within one organism provides changes in environment. It is very likely that transmission bottlenecks have been a major driving force in generating HIV diversity [41,98].

Progress in the development of synthetic vaccines has been slow. Proteins or oligopeptides present the immune system with a more limited repertoire of relevant epitopes than the entire virus particle. Then, for statistical reasons, the problems summarized in previous paragraphs are aggravated: the level of immunity is lower and the chances of selecting variant viruses able to escape neutralization by the suboptimal immune response are increased. In the successful development of a hepatitis B vaccine (review in [268]) it became clear that viral particles or core structures afforded a better protection than an individual protein or synthetic peptides. In the course of studies to develop new anti-FMD vaccines, several protein or peptide constructions have been engineered that induce an enhanced response: amino acid bridging of two synthetic peptides [269], incorporation of a peptide epitope into viral core structures [260], and inclusion of foreign helper T-cell determinants [271]. It is likely, however, that important elements of the global response needed to afford protection are omitted by greatly optimizing particular epitopes. Very important new knowledge and progress has been derived from such efforts. Yet, it has not been possible to substitute whole virus-inactivated anti-FMD vaccines by synthetic vaccines.

7.2 Is there a viable strategy?

The antigenic diversity and instability of RNA viruses is best interpreted as a consequence of the *quasi-species* nature of viral populations and modifications in the equilibrium distributions of genomes (section 4). From the emerging data of crystal structures of antigen-antibody complexes [273], it appears that conservative amino acid substitutions (likely to be well represented in the viral *quasi-species* because they will hardly affect virion fitness) at key sites may diminish the affinity of antigens for antibodies. The factors likely to affect vaccine efficacy are: (i) the *rate of fixation* of amino acid replacements in virus-coded proteins (not directly the mutation rate as defined in section 2); (ii) the *number* of different epitopes involved in triggering the humoral and cellular immune response leading to protection; (iii) the *tolerance* of such epitopes for amino acid substitutions that result in a decreased affinity for antibodies or cells from the immune system. An added difficulty is the polymorphism of molecules of the major histocompatibility complexes through which most antigen-specific T-cells recognize antigens. The allelic form of such molecules influences which amino acid sequences are recognized by the immune system. The viral epitopes recognized as T-cell inducing determinants vary among individuals from one population [274,275]. This has led to some pessimistic views on the feasibility of vaccines based on T-cell inducing epitopes [274].

Since in spite of the above problems, some classical vaccines have been successful, the conclusion appears to be that the less epitope-specific, i. e., "the less synthetic" a synthetic vaccine looks to the immune system, the higher its chances of inducing protection. A new antiviral vaccine formulation should include: (i) a wide repertoire of epitopes recognized by B and T-cells; (ii) a number of variant amino acid sequences for the important and variable epitopes; (iii) complex, structured epitopes in the form of anti-idiotypic antibodies or capsid structures. In addition, the effects of carriers and adjuvants should be cautiously tested since they may have enhancing or suppressive effects on the immune response. Furthermore, in the process of conjugation to a carrier macromolecule, the antigens may be altered [276].

By ensuring a response to several viral epitopes, chances of selecting variants resistant to one or a family of antibodies are enormously reduced. This "multisite" response has an aim parallel, in principle, to

combination therapy with antiviral drugs (section 6.4). Partial immunity or biased immune responses will favor selection of antigenic virus variants. It must be emphasized, however, that selection of mutants resistant to "multisite" responses cannot be excluded; simply, their probability of occurrence is lower (see also section 8). For synthetic vaccine development, the animal protection tests should use not only the homologous virus for challenge, but a collection of natural variants likely to be present (or to arise) in the natural population of the virus which spread is to be controlled (see ref. [136]).
When possible, and for groups at high risk of severe disease, a combination of prevention by vaccination and antiviral therapy has also been suggested. Much research is needed to develop any such strategies, and whether they ultimately will prove successful is an open question.

8 Error-prone polymerase subsets, hypermutability, and error catastrophe. Is there a limit to RNA virus variation?

A clustering of A → G transitions was observed by O'Hara *et al.* in RNA from a VSV DI [108]. They suggested that it might have been generated by error-prone viral polymerases. In some positions around the G residues selected for error oligonucleotide analysis (section 2) to measure mutation frequencies in VSV, remarkable heterogeneities were found [77,78]. Cattaneo *et al.* identified an extremely high level of U→C transitions in the M gene of viral molecules isolated from the brain of a child afflicted with measles inclusion body encephalitis [115]. Relative to a consensus sequence, about 50 % of the U residues within the M-coding segment were changed to C in that particular set of molecules. They suggested that a selective hypermutability event led to enhanced U→C transitions, but not to other substitutions [115]. Since viral polymerase genes are themselves subject to variation, polymerase subsets with decreased fidelity of template copying could generate highly altered molecules, that would allow a transient, accelerated evolution [108]. An alternative mechanism for the generation of hypermutated molecules is the temporary incursion of RNA replication into the error catastrophe zone, as defined by Eigen, Schuster and colleagues [49,164,277]. The maximum polynucleotide length (V_{max}) whose information may be stably replicated relates to the error rate per nucleotide ($1-\bar{q}$, with $\bar{q}$ being the average copying fidelity) and to the

selectivity (or superiority) (δ_0) of the master sequence over its mutant spectrum. The threshold relation is $V_{max} < \ln \delta_0/1-\bar{q}$. Near the error threshold, a replicating RNA population has the advantage of an abundant mutant spectrum [49,164,277]. Occasional transitions into error catastrophe and the consequent "melting of information" may occur by either decreasing the fidelity of copying or by decreasing the selectivity of the master. The result may be either an abortive, dead-end infection, or the rescue of a new *quasi-species* if the brevity of the stay within the catastrophe zone allows it. A decrease in master superiority could be brought about by an environmental change, such as cell-to-cell (non receptor-mediated) invasion of brain cells by MV. If a new *quasi-species* is "rescued" in the new environment, completely different molecules modified at selected genomic locations may arise due to the different (not absence of) selective constraints. In some of the above examples, the bias in the type of mutations produced could be due to the participation of a double-stranded RNA unwinding activity [277a].

In slowly progressing brain disease, the many years elapsed between the initial infection and the final stages of a persistent infection do not permit excluding that frequent replication bottlenecks occurred in which only one or a few molecules succeeded in being amplified. Highly mutated molecules present at the very tail of the mutant spectrum could have been selected, the system having never entered the error catastrophe zone. Similar founder effects may explain the extremely high rates of fixation of mutations in prolonged, persistent infections involving low amounts of virus [100]. Hypermutational events cause abrupt changes in RNA and, along with recombination, permit greater evolutionary jumps than the continuum of high mutability. Is there a limit to the variation of RNA viruses? Are possible drug- or antibody-resistant mutants a limited set of genomes, or are we isolating only a few out of a hopelessly large number of representatives? In a recent phylogenetic analysis of VP1 of 15 isolates of FMDV, it was shown that fixation of amino acid replacements had occurred at a limited number of residues, in relation to that expected from a Poisson distribution of changes [278]. The comparison was among a limited number of relatively recent FMDV isolates. It is becoming apparent, however, that as new isolates are entered into the comparisons, changes are increasingly found in previously invariant positions. As a recent example, the hemagglutinin of IV type B of isolate B/Ann Ar-

bor/1/86 showed six amino acid substitutions not found in 1940–1983 isolates, and each of the hemagglutinins compared had unique substitutions [279]. The same applies to the M protein of MV isolates [280]. Long-term evolution tends to preserve functional domains as defined by charge distributions and polypeptide foldings, rather than primary sequences. Thus, variations at one site on a viral polypeptide may demand "accompanying" changes at other sites to maintain functionality. Since several amino acids have similar chemical properties, the number of possibilities for compensating substitutions appears to be very large. An experimental analysis that supports this notion has come from the comparison of 31 reverse transcriptase sequences from different origins [281]. Only three out of about 300 residues were invariant in all the enzymes! With time, an enzyme that is essential for the replication cycle of retroviruses changed almost completely its primary sequence and yet maintained its function. Considering that even single, conservative amino acid substitutions may alter the behavior of an RNA virus (section 5), and that each infected organism includes distributions as shown in Figure 1B and C (section 3) (but with 10^9 to 10^{12} genomes with an ever-changing succession of distributions!), it may be concluded that the potential for genetic as well as for phenotypic variation of RNA viruses is indeed enormous.

9 Conclusion

RNA genetics deals with uncertainty and with probabilities [93]. Nucleotide sequencing is currently providing great insight into the detailed organization of the genetic material. Yet, RNA geneticists are becoming aware that each RNA virus molecule sequenced will most likely never be found again as an exact replica. This is becoming dramatically apparent in the sequencing of HIV [117,118, 281a]. However, it must be remembered that it is the very same phenomenon previously documented for many other RNA viruses, including retroviruses (sections 3 and 4). This individual indeterminacy of RNA genomes may be safely overlooked in some studies, but not in others. It will hardly affect studies such as the elucidation of three-dimensional structures by crystallographic methods or the gene expression strategy of a virus. Other important biological traits, however, are critically dependent on the fine, single-base residue or single amino acid residue make-up of the virus. Cell tropism and host-range, attenuation or virulence, affini-

ty for antibodies, recognition by cells of the immune system, and resistance to antiviral agents are some of the important phenotypic traits critically dependent on single-residue variations [37,41,45,93]. It is for such biological properties that the uncertainty of relating a *defined* nucleotide sequence to an observed behavior becomes apparent [93]. RNA heterogeneity is directly relevant to viral pathogenesis. Infection of a susceptible organism can be viewed as a succession of invasions of cells and tissues modulated by a series of responses from the organism. It is a very adequate playground for the continuous generation of new genomic distributions, even though the same *average* genome may be repeatedly selected in the same tissue or organ, or intact organism or populations of organisms.

The above considerations make it very unlikely that RNA viruses evolve simply by a steady accumulation of mutations. Instead, unpredictable shifts of genomic distributions, along with very frequent transmission bottlenecks are the main driving evolutionary forces. It may be a matter of chance that the comparison of viral sequences belonging to isolates distant in time may appear as a gradual accumulation of mutations. It may also be the result of an averaging of rapid evolution events along with periods of stability, as predicted by the population equilibrium model [93] for RNA genomes (section 4) and by the punctuated equilibrium model of evolution generally. As a consensus distribution, a virus may remain relatively invariant for thousands of years [42] or undergo exceedingly fast change in *one* infected individual [100]. "New" diseases such as human hemorrhagic conjunctivitis [139], and AIDS or the adaptation of a morbillivirus to seals [282–284] can be viewed as examples of rapid genetic and biologic shifts in RNA genomes. As a consequence, dealing with the evolution of RNA viruses required a theoretical framework different from the classical phylogenetic methods consisting in the derivation of rooted trees. At present, the most adequate theoretical study is provided by the concepts of *quasi-species* as developed by Eigen, Schuster and colleagues (section 4).

In a certain sense, RNA viruses appear as "collections of entities waiting to be selected for", and perhaps that is why they have been successful parasites all along.

10 Summary

RNA viruses and other RNA genetic elements must be viewed as organized distributions of sequences termed *quasi-species*. This means that the viral genome is statistically defined but individually indeterminate [76]. Stable distributions may be maintained for extremely long time periods under conditions of population equilibrium. Perturbation of equilibrium results in rapid distribution shifts. This genomic organization has many implications for viral pathogenesis and disease control. This review has emphasized the problem of selection of viral mutants resistant to antiviral drugs and the current difficulties encountered in the design of novel synthetic vaccines. Possible strategies for antiviral therapy and vaccine development have been discussed.

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While this review was in the press, Heinz *et al.* (J. Virol. *63* [1989] 2477) reported that human rhinovirus 14 mutants with high resistance to WIN 52084 (Fig. 7, section 6.1) occur at frequencies of 4×10^{-5} and those with low level resistance are found at frequencies of 1×10^{-3} to 4×10^{-4}, in excellent agreement with current estimates of mutation frequencies for RNA viruses (section 2).

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