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RAPID EVOLUTION OF RNA VIRUSES

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INTRODUCTION

Viruses with RNA genomes are the most ubiquitous cellular parasites known. They are found intracellularly in nearly all life forms from plants and animals to fungi and prokaryotes. They are diverse in size, structure, genome orga-

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nization, and replication strategy, and have been classified into several groups based on these and other criteria (106). RNA viruses have been the object of extensive study because they are responsible for a variety of medically and economically important diseases of man, plants, and animals. Many RNA viral infections, however, are inapparent or asymptomatic, causing little or no obvious detriment to the host. The extraordinary evolutionary success of RNA viruses is attributable to their ability to use different replication strategies and to adapt themselves to the widely varying biological niches encountered during virus spread in a single host or multihost network.

RNA viruses have been recognized as highly mutable since the earliest studies. In the 1940s Burnet & Bull (22) observed phenotypic changes associated with passage of influenza A virus in chick embryos, and Kunkel (95) reported early evidence for the mutability of plant viruses from studies with tobacco mosaic virus (TMV). Subsequent genetic studies on TMV (112), Newcastle disease virus (61), phage Qβ (160), reovirus (53), and vesicular stomatitis virus (126, 127) provided further evidence of RNA genome instability.

In recent years modern techniques in molecular and cellular biology have allowed detailed study of natural virus isolates and of defined laboratory virus strains. Emerging from these studies is a view that RNA virus populations are extremely heterogeneous, which allows for great adaptability and rapid evolution of RNA genomes. In this review we discuss evidence for the heterogeneous (quasispecies) nature of RNA genomes, some factors influencing their rapid evolution, and biological implications of this rapid evolution. The large volume of relevant subject matter and references cannot be covered in detail in this review. For further details and references several other recent reviews are available covering RNA virus evolution (14, 41, 42, 75, 132) as well as evolution of influenza viruses (122, 164), poliovirus (90), and AIDS (HIV) viruses (30).

RNA VERSUS DNA GENOMES

All known cellular organisms use DNA genomes for storage of genetic information. RNA genomes, although they probably preceded DNA as the original information carriers (49), are now found only in host-dependent cellular parasites (e.g. RNA viruses, viroids, yeast killer plasmids). The reason for this is obvious; DNA is more stable than RNA, and owing to the elaborate proofreading and repair mechanisms that have evolved, DNA is replicated with much greater fidelity.

Rates of Evolution

Rates of evolution have now been calculated from available sequence data for several cellular genes in a number of different animal species. The rates of

neutral mutation vary among genes and among species, but average less than 10^{-9} substitutions per site per year (19, 100).

Rates of evolution of continuously replicating RNA viruses may exceed by a million fold the rates of evolution of their DNA-based hosts. Nottay et al (116) reported mutation of 1–2% of the poliovirus genome during the 1-yr spread of an outbreak in 1978. Buonagurio et al (21) sequenced the NS gene of influenza A viruses isolated over 50 years. They found a uniform rate of evolution of about 2×10^{-3} substitutions per site per year. The rate of evolution of the AIDS virus has been estimated (66) to be between 10^{-2} and 10^{-3} substitutions per site per year for the *env* gene (that for *gag* is about 10-fold lower).

Gojobori & Yokoyama (59) compared rates of evolution for the v-*mos* gene of Maloney murine sarcoma virus with those for its cellular homolog, c-*mos*. The rate of nucleotide substitution was estimated to be 1.31×10^{-3} per site per year for v-*mos* versus 1.71×10^{-9} for c-*mos;* again, a millionfold difference was demonstrated in rates of evolution of RNA versus DNA.

Polymerase Fidelity

Obvious reasons for the differences in evolution rates of DNA and RNA are the shorter generation times for replicating RNA viruses and the error-prone nature of viral RNA polymerases compared to DNA polymerases.

Mutation rates for DNA genomes have been estimated to be between 10^{-7} and 10^{-11} per base pair per replication (46, 56). Drake (46) observed that in prokaryotes genome size was inversely proportional to mutation rates. He suggested that larger genomes must pay the energy costs of increased fidelity (proofreading) and repair in order to produce viable genome copies, while smaller genomes would not require the same fidelity and would not need to evolve energy-consuming mechanisms which would limit variability. The fidelity of DNA polymerases measured in vitro varies significantly with enzyme, template, and conditions (56) and generally does not reflect estimated in vivo fidelity, perhaps because of differing degrees of proofreading in the in vitro reactions.

Error frequencies have been estimated by different methods for several RNA polymerases (see below). Estimates vary, but most average about 10^{-4} errors per site following a limited number of replications. The limited fidelity of RNA polymerases reflects the lack of effective proofreading mechanisms. Although evidence for 3' →5 ' excision has been reported for influenza virus polymerase (81), and although 3' →5 ' pyrophosphorylytic excision has been documented with T4-modified RNA polymerase (85), corrective proofreading of elongating RNA has not been demonstrated.

Interestingly, DNA mutation rates of immunoglobulin variable region genes can be as high as 10^{-3} per cell generation during some stages of B-cell development (116a, 145a, 162). Perhaps when mutation is desirable to gener-

ate variability, proofreading mechanisms are suppressed or bypassed and fidelity of DNA replication becomes comparable to that of RNA.

RECOMBINATION OF GENETIC INFORMATION IN RNA VIRUSES

In addition to stepwise evolution due to the accumulation of point mutations, RNA viruses have available to them mechanisms of reassortment and recombination that allow for large-scale evolutionary jumps. True intermolecular recombination, which is a major means for DNA evolution, occurs frequently with certain RNA viruses but may occur rather infrequently with others.

Genome Reassortment of Segmented RNA Viruses

A number of RNA virus groups have evolved multipartite or segmented genomes. Each segment contains a viral gene (or genes), and the full complement of segments is normally necessary for virus-particle infectivity. Mixed infection of a host cell by different strains of a virus then allows for the rise of progeny viruses with differing subsets of genome segments from each parent.

Keroack & Fields (89) and Tyler et al (159) have used reassortment experiments to identify reovirus gene segments responsible for various viral functions and traits (see also references in 89, 159). Allen & Desselberger (2) demonstrated the utility of reassortant rotaviruses for amplifying segments of poorly growing human rotavirus in bovine rotavirus recombinants that can replicate efficiently. Shope et al (145) used reassortment to correlate bunyavirus pathogenicity in mice with the M (middle size) RNA segment.

In several examples, e.g. influenza A strains (140) and reovirus (136), low-virulence parental viruses have given rise to highly virulent reassortants. Recently Riviere & Oldstone (133) studied the pathogenesis of reassortants from three parental strains of lymphocytic choriomeningitis virus (LCMV), an arenavirus that has two genome segments. Two of the reassortants caused lethal disease in mice, while parental strains and reciprocal reassortants did not. Some virulent pandemic episodes that are caused periodically by influenza A virus result from reassortment of human strains with avian, animal, or other human strains, which generates antigenically novel virus (13, 36, 52, 164, 166).

Recombination in RNA Virus Genomes and Defective Genomes

RETROVIRUSES Retroviruses are unique among the RNA viruses in that they replicate via a DNA intermediate, which can be integrated within the host-cell chromosome. This allows these viruses the luxury of two distinct

life-styles: that of a replicating RNA virus with inherent high mutability, and that of a stably integrated provirus that is replicated only infrequently by high-fidelity DNA replication machinery.

Mutations can be generated by a variety of mechanisms during retrovirus replication. Point mutations occur at a high frequency during reverse transcription (12, 34, 60). Deletions, insertions, duplications, and other rearrangements can also occur during reverse transcription, as can recombination with other viral (or cellular) sequences (45, 119, 144). Such recombinations are most likely generated by template switching or by a copy-choice mechanism (29). Recombination can also take place between unintegrated viral DNA and integrated provirus DNA, defective provirus DNA, or cellular DNA with sequence homology. The transcription of retroviral genomic RNA by cellular RNA polymerase can be another major cause of mutation.

Retroviruses, retrotransposons, and related elements, because of their unique replicative mechanisms, may have significant effects on the evolution of eukaryotic genomes (7). Processed pseudogenes and certain highly reiterated DNA sequences, which make up over 5% of the human genome, probably arose by reverse transcription of cellular RNAs (7). The discovery that the transposable Ty element of yeast (15) and copia-like elements in *Drosophila* (55, 138) are related to retroviruses raises the possibility that retroviruslike elements are ubiquitously scattered throughout eukaryotic genomes. For example, representatives of the L1 family of highly repetitive DNA sequences, which are found throughout mammalian genomes, contain sequences with homology to known reverse transcriptases (70, 101). Transposition mediated by these types of elements may profoundly affect host-genome evolution by promoting recombination and insertional mutagenesis, and more importantly by dispersing endogenous promotor/enhancer regions throughout host genomes.

STANDARD RNA VIRUSES Molecular recombination for generation of virus diversity has been demonstrated only for a limited number of nonretroviral RNA viruses.

For many years true intermolecular RNA recombination (in nonretroviruses) could be demonstrated only for picornaviruses (32, 92). Recombination of genome RNA occurs frequently during picornavirus replication, apparently by template switching during negative-strand synthesis (93). In tissue culture, recombinants between isogenic strains of foot-and-mouth disease virus were detected at a frequency of 0.92% (107), and intratypic poliovirus recombinants were seen at a frequency of 0.13% between genetic markers separated by only 190 bases (93). Frequencies of recombination between less closely related strains of virus are much lower (93, 156). However, intertypic poliovirus recombinants have been readily isolated from humans vaccinated

with the three Sabin attenuated strains (111) and from close contacts of vaccinees (90). Indeed, Minor et al (111) isolated two distinctly different recombinants from the same vaccinee. These vaccine recombinants appear to represent variants that partially escape the immune responses of the vaccinees.

Lai and coworkers recently demonstrated that homologous recombination of genomic RNA occurs in coronaviruses (96), apparently at a high frequency (105). The discontinuous replication of coronaviruses generates template-free intermediates containing nascent RNA strands complexed with polymerase. These intermediates bind (by specific base pairing) to new templates, generating copy-choice recombinants.

Recombination has also recently been reported for the plant virus brome mosaic virus (20). This virus has a positive-strand tripartite genome with a conserved stem-loop structure at the 3' end of each segment. Deletions in this region of one genome segment were repaired during infection (with restoration of the stem-loop structure) by both homologous and nonhomologous recombination with the 3' ends of unaltered gene segments.

Intermolecular recombination in negative-strand RNA genomes is much less common. One clear example is a mosaic defective interfering RNA of influenza (84), but this and other examples of intermolecular recombination show no base-pairing specificity in the nascent strand–polymerase leaps to new templates (i.e. are generally nonhomologous). Therefore, generation of viable infectious intermolecular recombinant genomes of negative-strand viruses may be rare. However, even extremely rare recombination events may have a significant role in RNA virus evolution. Recombinant viruses of another type, defective interfering (DI) particles, have been demonstrated for nearly every type of animal virus. The generation of nearly all DI particles can be explained by some variation of copy-choice intramolecular recombination (78, 98, 99, 124). The significance of DI particle generation is discussed later in this review.

HETEROGENEITY OF RNA GENOMES

The Quasispecies Concept

Eigen and coworkers developed the quasispecies concept to describe the distributions of self-replicating RNAs, which they proposed were the first genes and the precursors to life on earth (48–50). They noted several reasons to believe that RNAs preceded DNA as information carriers. In cells DNA monomers are synthesized via ribose intermediates, DNA replication is initiated on RNA primers, and information is processed by RNA-protein machinery (49). The fact that RNA can fold into a large variety of three-dimensional structures and the recent findings that RNA can have cat-

alytic properties (63, 168) further strengthen the argument for RNAs as the first genes.

The ability to self-replicate may have been unique to RNA in a prebiotic soup. Inoue & Orgel (80) have demonstrated that under appropriate conditions template-directed RNA synthesis will occur in the absence of enzyme. Prebiotic, nonenzymatic RNA synthesis of this type would have proceeded with very high error frequencies, on the order of 10^{-1} to 10^{-2} (50, 80). The error threshold probably limited the maximum length of early self-replicating RNAs to between 50 and 100 bases (49). (The error threshold of RNA synthesis through evolution has similarly limited the length of present-day RNA genomes and cellular mRNAs.) The distribution of early RNAs was such that sequences that were most fit (i.e. those that were most stable and that had superior replicative ability) would have been present in higher concentrations. However, replicative infidelity would have ensured a large proportion of related nonidentical sequences. Eigen and colleagues (49) stated that "the result of self replication competition had to be the master sequence together with a huge swarm of mutants derived from it and from which it had no way of escape." They termed this mutant distribution a "quasispecies" distribution.

Evidence for a Quasispecies Distribution of Genomes in Present-Day RNA Virus Populations

BIOLOGICAL EVIDENCE There have been numerous documentations of heterogeneity in RNA virus populations. We cite only a few examples of extreme heterogeneity in virus populations of known origin and history.

The first recognition that RNA viruses might have quasispecies distributions was made in Wiessmann's laboratory with phage Qβ (11, 40, 43). Domingo et al (43) showed by T_1 fingerprinting that individual clones isolated from a multiply passaged Qβ stock virus differed from the stock virus by an average of one to two nucleotides, in spite of the fact that the stock-virus fingerprint had remained unchanged over the previous 50 passages. In competition experiments, variant clones were consistently outgrown by parental wild-type virus. Domingo et al concluded "that the phage population was in a dynamic equilibrium with viable mutants arising at a high rate on one hand and being selected against on the other," and added, "the genome of Qβ phage cannot be described as a defined structure, but rather as a weighted average of a large number of individual sequences." They further pointed out that the relative growth rate of a variant must be extremely close to that of the wild type (within 0.01–0.1%) in order to be detected by ordinary sequencing of the equilibrium population.

Several sequencing studies have demonstrated extreme heterogeneity of viral populations. Fiers and coworkers observed sequence heterogeneity when phages Qβ and MS2 were first sequenced (37, 84a). Variability in several

virus populations has been suggested by sequence differences among overlapping cDNA clones generated from viral RNA (4, 54, 58, 141). Catteneo et al (24) reported over 1% sequence difference among overlapping cDNA clones of measles virus RNA isolated from brain tissue of a single subacute sclerosing panencephalitis (SSPE) patient. Hahn et al (66) recently sequenced the *env* gene of AIDS virus isolates collected at different times from a maternally infected child. All isolates differed, and the differences suggested that they had not evolved sequentially, but had diverged from a common ancestor.

Heterogeneity has been detected in populations of foot-and-mouth disease virus from single natural isolates (39, 135) and after replication in cell culture (147). Poliovirus shed by vaccinated individuals can be extremely variable (90, 91, 111). Indeed, one study (91) reported evidence that over 100 genetic changes occurred during vaccine virus replication in one or two individuals. Likewise, Sabara et al (137) detected heterogeneity among each of six natural isolates of bovine rotavirus examined. Recently several cocirculating antigenic variants of influenza A were isolated during a single epidemic in a semiclosed community (121); similar variation had been observed earlier with influenza B (120). The investigators suggested that the epidemics were probably initiated by single individuals' excretion of a mixture of antigenic variants, which were differentially selected for in subsequently infected individuals.

QUANTITATIVE EVIDENCE Relatively few experiments have been performed to measure RNA virus polymerase error frequencies (or mutation frequencies) during a limited number of virus replication cycles.

The reversion rate for one extracistronic point mutant of phage Qβ was calculated to be about 10^{-4} per genome doubling (11, 40). The mutation frequency at one site in the Rous sarcoma virus genome was estimated to be about 3×10^{-4} per virus passage (31), which is consistent with high in vitro error frequencies demonstrated with reverse transcriptase of avian myeloblastosis virus (12, 60).

Steinhauer & Holland (153) recently developed a technique for direct measurement of the polymerase error frequencies at selected G residues in viral RNA. Error frequencies at one highly conserved site in vesicular stomatitis virus (VSV) exceeded 10^{-4} in clonal pools of virus replicated in vivo and in transcription products in vitro. Similar error frequencies have been observed repeatedly at two other sites of the VSV genome that have been extensively characterized. Significantly, at one site at which two of the three possible mutations generate lethal stop codons in the N gene a similarly high error frequency was also determined (D. A. Steinhauer & J. J. Holland, unpublished).

Heterogeneity has also been demonstrated in single plaques of influenza A

virus. Parvin et al (123) sequenced the NS gene from multiple individual virus plaques that were generated during growth of a single plaque. Of approximately 92×10^3 bases sequenced, seven mutations were detected, all of which were neutral with respect to growth kinetics. Lethal or very debilitating mutations would not, of course, have been scored in this study. Interestingly, when Parvin et al did the same experiment on a segment of the VP1 gene of poliovirus type 1, no mutations were detected in over 95×10^3 bases sequenced. The authors calculated rates of neutral mutation of 1.5×10^{-5} for influenza virus and less than 2.1×10^{-6} for poliovirus at these sites (assuming five replication cycles during growth of each plaque). The reason for the lower rates for poliovirus could be greater restraints on VP1 or differing polymerase error frequencies or purifying selection difference, but it is not possible at present to distinguish among them.

Durbin & Stollar (47) recently reported a remarkably low error frequency (less than 5×10^{-7}) for the reversion of a particular Sindbis virus host-restricted mutant. It would be interesting if certain viral RNA sites are replicated with much higher fidelity than others, and the molecular basis for this would require investigation. Stec et al (152) observed that Sindbis virus variants resistant to monoclonal antibodies arose spontaneously at frequencies between $10^{-3.5}$ and 10^{-5}; thus it is possible that the reversion rate of Durbin & Stollar (47) might have reflected the combined frequencies of two complementing mutations, one of which was not in the sequenced region of the genome. In any case, much more work is needed to quantitate mutation rates and polymerase error frequencies at defined sites for a variety of RNA viruses (including retroviruses and retrotranscribing DNA viruses such as hepatitis B virus).

HIGH MUTATION RATES VERSUS RAPID RATES OF EVOLUTION

Wild-Type and Relatively Stable Equilibrium Populations

In spite of great heterogeneity within virus populations, high mutation rates are not always reflected in rapid evolution. There are conditions under which viruses can replicate efficiently and continuously, and yet accumulate few if any viable, competitive mutations in the virus population. The studies of Domingo et al (43), with phage $Q\beta$ first illustrated this. The same wild-type sequence predominated through extensive passage despite the fact that a large portion of the population was shown to be variant at any one time. Once a stable equilibrium population has been reached, virus may be able to replicate for extensive periods with little evolution as long as conditions remain unchanged. This does not mean that the predominating sequence cannot stray to some degree from the master sequence or that a rare event (many mutations at one time, or a recombinational event) could not give rise to a more fit

variant (a jump from one fitness peak to another). It simply means that among the distribution of variants generated by the wild-type sequence, none have a competitive advantage over the parental master sequence.

Observations with VSV support this conclusion. Laboratory strains of VSV with different passage histories over many years accumulated few if any nucleotide changes as revealed by T_1 fingerprinting (28). Spindler et al (148) fingerprinted isolates obtained during 232 dilute passages of VSV in BHK_{21} cells. Although several spot changes were seen in intermediate passages, all reverted to wild type by passage 232. Recently, after 529 dilute passages of this virus, T_1 oligonucleotide mapping again revealed the accumulation of not a single oligonucleotide spot change (D. A. Steinhauer & J. J. Holland, unpublished).

Other viruses also demonstrate remarkable stability in some situations. The type 3 Sabin poliovirus vaccine differed from its neurovirulent progenitor at only 10 nucleotide positions after 53 in vitro and 21 in vivo passages in monkey tissue (150). In 1977 H1N1 influenza A virus reappeared in the human population after 27 years of dormancy with sequences nearly identical to those of the 1950 virus (88, 114). It is possible that virus was harbored in a nonhuman host where it remained very stable. Alternatively, virus may have remained viable in a nonreplicating state (frozen or desiccated) before reemergence. It is significant that the H1N1 sequences evolved rapidly and in divergent directions in subsequent human outbreaks (130). Rotavirus genome exhibited only limited heterogeneity after years of continuous passage (4).

Many selective forces may stabilize virus populations. These stabilizing factors may include the need for conservation of protein structure and function, RNA secondary structure, glycosylation sites, and phosphorylation sites. Even third-codon changes can be subject to selective pressures (62). Recently, remarkable conservation of certain protein domain sequences has been observed between completely unrelated RNA viruses (1, 3, 69). These observations argue for a modular theory of evolution (16) that suggests that selective forces may often act at the level of subgenomic functional units rather than solely upon intact virus particles.

Conditions That Promote Disequilibrium Favor Rapid, Random Evolution

Any change in environmental conditions that offers variants in a virus population the opportunity to compete favorably with the predominating virus can shift the equilibrium and drive virus evolution. The countless factors that can contribute to disequilibrium include interference by DI particles, different host or cell types, immune selection, transfer from vectors to hosts or vice versa, and temperature shifts. During VSV infection of BHK cells, where low multiplicity passages favored the establishment of a very stable equilibrium population (see previous section), high multiplicity passages led to rapid and

random genome evolution (148). Similarly, rapid evolution of the VSV genome was also seen in persistently infected cells (72). In these instances virus evolution was partly driven by DI particles. DI particles are amplified during high multiplicity passages and interfere with parental virus replication. This forces the evolution of standard virus mutants (Sdi^-) that are resistant to interference by the predominating DI particles (76, 77). It appears that continuous coevolution takes place at the termini of virus and DI genomes and the replication-encapsidation proteins with which they specifically interact (35, 117). Poliovirus also demonstrates reasonable genome stability in some situations (51, 150), but can evolve extensively upon replication in the human gut (90, 91, 111). This is reflected in the rapid evolution seen as poliovirus outbreaks spread in human host populations (104, 116). Likewise, Takeda et al (155) documented continuous and divergent evolution of enterovirus 70 genomes from about its time of appearance in Africa in the 1960s through two pandemics of acute hemorrhagic conjunctivitis.

Host immune selection can be strongly involved in driving virus evolution. This is clearly demonstrated with influenza virus. Antigenic variants are regularly selected, so previously immune hosts are no longer protected against newly circulating strains (122, 164). Other factors, including reassortment of independently evolving modular genome segments, are important in the rapid evolution of influenza A viruses. Influenza viruses have been reported to exhibit rapid and continuous evolution in vitro even in conditions in which other mutable viruses such as VSV (18) and poliovirus (123) do not evolve as extensively.

Salinovich et al (139) recently demonstrated strong evidence for immune selection of antigenic variants with the lentivirus equine infectious anemia virus (EIAV). Persistent infection by EIAV is characterized by periodic emergence of disease. Isolates from four consecutive disease episodes of a persistently infected pony revealed a novel predominating variant each time. Although the variants were closely related, as shown by T_1 fingerprinting, each variant was distinguishable antigenically by neutralization assays with the pony's serum and by Western blot analysis using monoclonal antibody to the major surface glycoprotein. Antigenic variants can also be selected during infection by visna virus (27) and probably HIV, the AIDS virus (151). The role of these variants in persistence is unclear (64). Cellular immune selection can also drive rapid evolution of viral RNA genomes. Extensive mutations in the G protein (and fewer in M and NS proteins) were observed in variants of VSV selected in vivo by natural killer cell lysis of persistently infected cells (161).

Acute Versus Persistent Infection

RNA viruses that normally cause acute infection in susceptible hosts can sometimes cause a long-term subacute infection. Persistent infection can be

initiated by factors such as multiple mutations, interferon, defective interfering particles, host cell properties which prevent efficient virus replication or maturation, etc. (74, 167). Maintenance of the persistent state requires only that some balance be reached between virus replication and host function, and possible viral etiology is suspected for a number of long term degenerative and autoimmune diseases.

Subacute sclerosing panencephalitis is a rare but fatal degenerative disease of the central nervous system resulting from persistent measles virus infection; the disease becomes manifest years after acute infection. In the CNS neurons of SSPE patients, intracellular replication of nucleocapsids continues, but no mature budding virus particles are detected. Several studies suggest that a defect in M (matrix) protein expression is involved (67, 68). Experiments utilizing cells derived from brain tissue from SSPE patients (23) and direct observation of brain from SSPE patients (5, 65) showed a block at the level of M gene mRNA translation. Other workers (143) observed synthesis and then rapid degradation of M protein in one cell line derived from the brain of an SSPE patient. Another study reported a stop codon within the M gene coding region of RNAs isolated from SSPE-patient brain (24). There is no reason to believe that only defects in the M gene are associated with SSPE. Norrby et al (115) have detected M proteins in the brain of some SSPE patients, and Baczko et al (6) have suggested that SSPE can be correlated with defects in other measles genes or gene products which prevent normal virus particle assembly. Haase et al (65) demonstrated repressed synthesis of all viral RNAs in SSPE-affected brain and suggested that generalized constraints (of an unknown nature) on virus gene expression may be important in establishing persistent CNS infections by a variety of viruses.

Whether virus genome degeneration is a cause or a result of SSPE is not clear, but extreme variability has been demonstrated among viral RNAs present in infected brain. Catteneo et al (24) detected as much as 1% sequence variation in the M gene of overlapping cDNA clones from the same area of the same SSPE-patient's brain. They suggested that extreme variability could be responsible for the observed diversity of measles virus gene expression in SSPE patients. This heterogeneity is not unexpected, since long-term intracellular replication of measles virus RNA probably offers reduced opportunity for selection against mutants in the M, F, and H proteins, which are mainly involved in virus maturation, release, and reentry.

Paget's disease is a chronic bone disorder that affects nearly 4% of the human population aged 70 and over. Viral antigens of a number of paramyxoviruses, including measles virus, respiratory syncytial virus, simian virus 5, and parainfluenza virus type 3, have been detected in bone tissue of patients with this disease (10, 109). Recently measles virus RNA was detected by in situ hybridization to bone tissue of a patient with Paget's disease (9), but any role of these RNA viruses in Paget's disease is obscure as yet.

Several viruses have been loosely linked to autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and juvenile-onset diabetes, but definitive cause and effect is difficult to assess. Virus infections are known to trigger immune responses which can sometimes cross-react with host tissue (57). Srinivasappa et al (149) demonstrated that cross-reactivity of antiviral antibodies with normal tissues is fairly common by screening 14 organs with 600 monoclonal antibodies raised against 11 viruses. Although molecular mimicry may arise frequently in rapidly evolving viruses, it probably only rarely triggers serious diseases. Onset of disease would depend upon other factors such as strength and duration of autoimmunity, major histocompatibility complex (MHC) types, and type and strain of virus.

BIOLOGICAL IMPLICATIONS OF VIRAL RNA GENOME PLASTICITY

Antigenic Change and Vaccine Problems

Very rapid evolution of RNA viruses can create serious problems for vaccine design. Recent advances in molecular virology including the use of synthetic peptides, recombinant viral vectors, and cDNAs as vectors or stable seed inoculum should ensure the effectiveness (and safety) of future vaccines. The challenge will be to design safe vaccines that can elicit strong immune responses and yet be maximally effective in the wake of antigenic changes during virus evolution.

The large number of monoclonal antibodies now available for RNA viruses has allowed studies of antigenic variation in defined populations as well as among isolates collected at different times and locations and from different hosts. The frequency of resistance to monoclonal antibodies can vary greatly (102), but averages between 10^{-4} and 10^{-5} (41, 75, 125). Most viruses exhibit strongly conserved, moderately conserved, and poorly conserved antigenic sites in their surface protein; an ideal vaccine might target strongly conserved epitopes, but this is not always possible. Multivalent vaccines and frequent modification of vaccines may be needed to deal with rapid virus evolution in many circumstances.

Vaccines that elicit a cellular immune response as well as an antibody response may often prove most effective. Cytotoxic T-cell responses are not limited to subtype specificities as defined by antibody epitopes. Polyclonal murine cytotoxic T cells raised against respiratory syncytial virus showed cross-reactivity with all human virus subtypes tested (8). Subtype cross-reaction of Tc cells was demonstrated earlier with influenza A virus (158, 165). Broad T-cell reactivity is to be expected, because cytotoxic T cells can recognize not only virus surface antigens on influenza A–infected cells, but also fragments of the internal nucleocapsid protein presented at the cell surface together with MHC antigens (157, 165). Similarly, with VSV the N

protein was demonstrated to be the major antigen recognized by cytotoxic T cells (128). Variability in T-cell target sites remains to be studied, but it is probably lower than that of surface-antigen epitopes.

Other problems for vaccine design include the masking of antigenic determinants by oligosaccharides on enveloped virus glycoproteins (44, 146) and possibly the protection of important regions on surface proteins by functionless antigenic domains that are free to drift without constraint, as proposed for HIV (AIDS) viruses (30).

Even in well-vaccinated populations RNA virus variants are likely to arise at intervals and cause outbreaks of disease. Novel antigenic variants recently caused a small poliovirus outbreak in Finland, where 97% of the population had been vaccinated (104). Sequencing showed that an isolate from the outbreak was related to type 3 polioviruses but significantly different in areas known to be antigenically important (79). The isolated virus was probably not derived from vaccine, but from circulating wild-type virus.

The long-term success of the poliovirus vaccines, as contrasted with the need for frequent updating of the antigenic composition of influenza A vaccines, illustrates clearly that any RNA virus vaccine program must take account of the relative degree of antigenic plasticity (or constraint) involved in the targeted viral epitopes. When these epitopes tolerate extreme variability, effective vaccine design will pose greater challenges, and continuing modification may sometimes be required.

Host-Cell Specificities and Changes in Disease Patterns and Virulence

A major factor determining host-cell specificity is the recognition by virus capsid or glycoprotein surfaces of a specific protein or other receptor structure on the surface of susceptible cells. For example, HIV (AIDS) viruses have been reported to be specific for cells bearing the T4 (CD4) antigen (33, 94, 103). It was demonstrated long ago that poliovirus can replicate in refractory cells if virus genome is introduced intracellularly and thus bypasses the discriminatory receptor-binding step (73). Recently the human poliovirus receptor gene was cloned and transformed into poliovirus-resistant mouse L cells, whereupon they became sensitive to all three poliovirus types (108). Broad–host range viruses use cell-surface components that are common to many cell types and species.

Mere entrance into a cell does not ensure that virus will generate a productive infection. The cell must also allow for proper transcription, replication, and translation of viral nucleic acids and proper maturation of progeny virus. In the retroviruses, host-range determinants can be defined not only for the *env* gene (17, 45) (the likely receptor-binding site), but also for

the U3 region at the 3' end of the genome (25, 26, 97, 154). This site contains enhancer sequences that regulate viral RNA transcription in a tissue-specific manner (25).

Regardless of the molecular factors that determine cell, tissue, and species specificity, it is obvious that extremely rapid evolution of RNA genomes will on rare occasions result in marked changes in virus species specificity or tissue tropism and disease patterns. Sometimes only one or a few mutations can cause profound alterations in virulence phenotype or host or tissue tropism of a virus. A few changes in the U3 region of myeloproliferative sarcoma virus are responsible for its expanded host range and disease specificity relative to those of its progenitor virus, Moloney murine leukemia virus (154). A single nucleotide change in the T3 hemagglutinin gene of reovirus type 3 was demonstrated to alter both its growth and its tropism in the CNS (87). Rogers et al (134) demonstrated that a single amino acid substitution in the influenza hemagglutinin can alter receptor-binding specificity. Naeve et al (113) showed with an avian influenza reassortant virus containing human influenza virus hemagglutinin that only two nucleotide changes were necessary for altered receptor binding and replication in ducks. Reagan et al (131) isolated a mutant of coxsackievirus B3, which acquired an alternate site of attachment to virus receptor on HeLa cells following passage in rhabdomyosarcoma cells.

Similarly, viral pathogenesis can be greatly affected by single nucleotide changes. Only a single T_1 spot difference was detected between fingerprints of diabetogenic and nondiabetogenic variants of encephalomyocarditis virus (129). During the influenza A outbreak that occurred in chickens in the eastern United States in 1983, the virus mutated from an avirulent form to a highly virulent variant, which caused up to 80% mortality. Webster and coworkers (163) showed that a single point mutation was probably responsible for the acquisition of virulence. Seif et al (142) and Dietzschold et al (38) correlated virulence of rabies virus with changes in a single site in the glycoprotein. Stanway et al (150) showed only ten sequence differences between the genomes of Sabin type 3 poliovaccine and its neurovirulent progenitor, and Evans et al (51) demonstrated that only one of those mutations, in the 5' noncoding region of the genome, was necessary for reversion of the vaccine strains to neurovirulence. RNA genomes, with their extreme mutation rates and great genetic plasticity, constantly threaten to undergo major or minor changes in tropisms and/or disease propensity.

Unpredictability of Future RNA Virus Disease Outbreaks

To the extent that we associate certain viruses with certain defined disease syndromes (e.g. measles virus with measles, poliovirus with polio, HIV with AIDS), we also tend to overlook the phenotypic plasticity and the inherent

unpredictability of all rapidly evolving RNA virus genomes. It is true that virus populations tend toward defined patterns of transmission and pathogenesis once they occupy a stable ecological niche. Hence, even a continuously drifting RNA genome may produce a specific disease syndrome in one preferred host for very long periods. Even when there is rapid and extensive genomic evolution (as in the case of influenza virus A), there may nevertheless be strong conservation of the overall structure and function of viral proteins. This conservation is to be expected, since it maximizes replication and transmission efficiency, virus stability, and recovery and survival of major host species. Conservation does not, however, mitigate the possibility of the rare appearance of new virus strains with markedly different structures, host ranges, tissue tropisms, replication and transmission strategies, and disease patterns and virulence. Variation will be particularly favored whenever new or expanded ecological niches present themselves to evolving virus populations. The global expansion of the human population during the last century is an obvious example of evolving opportunities for viral RNA genomes, particularly when the impact of human activities on other niches is considered (e.g. deforestation, agricultural development, growth of large cities, and construction of rapid transportation networks). Therefore it should not be surprising that previously unknown human, domestic-animal, and plant-crop diseases will appear at intervals, and that many or most will be due to evolving RNA genomes. Human AIDS is but one example of such a newly recognized human disease. Enterovirus 70 is a newly recognized virus that causes pandemics of acute hemorrhagic conjunctivitis in humans. It has diverged continuously since its appearance in Africa in the late 1960s, as documented by the oligonucleotide mapping studies of Takeda et al (155). Presumably this virus was derived from an animal (or human) virus that had not used this ecological niche or mode of transmission.

It is clear from sequencing data now available that despite the presence of some strongly conserved genomic stretches, well over half of the nucleotide positions in many RNA virus genomes can be substituted during their evolution without loss of virus viability. One consequence of this genomic plasticity is the statistical certainty that completely new RNA genomes will continually be generated. A 10-kb genome would be required to test the viability and competitive characteristics of at least 4^{5000} different sequence permutations to find all fit combinations. This is an extremely conservative number, because the total information content of viral genomes can increase and decrease. However, this "small" astronomical number assures that not even a very minute fraction of all possible RNA viruses can ever be produced and tested. More importantly, it also assures that the large size and rapid evolution of the biosphere's viral RNA genome pools will constantly produce genome permutations that have never before existed. The vast majority of these will be

nonviable or noncompetitive and will generally disappear. The vast majority of viable and competitive new viruses will be unremarkable in their biological properties. But humans will have to live with, and try to cope with, new RNA virus variants that have particularly destructive phenotypes. However, when destructive viruses such as the AIDS viruses appear at intervals, it should be remembered that the new virus is not in fact a virus. It is a rapidly evolving quasispecies population of RNA genomes, some of which have greater or lesser virulence. Over longer periods of time, less virulent quasispecies populations will inevitably prevail over those that destroy the hosts upon which they depend.

Modulation of RNA Virus Evolution by Defective RNA Genomes

The rapid evolution of RNA genome populations can be affected not only by viable infectious virus mutants generated during replication, but also by the defective nonviable genomes that also arise during RNA virus replication as a result of viral replicase error (71, 78, 98, 99, 124). DI particles can affect RNA virus evolution in several ways. First, defective genomes can act as helper virus–dependent evolving gene "modules" (16) that are able to tolerate extensive genome rearrangement and base substitution (e.g. 118). If rare recombination events return portions of these extensively altered gene segments to infectious particles they can, when viable, cause profound phenotypic changes. DI particles of RNA viruses are often able to modulate virus lethality for cells, thereby allowing persistent infections in which virus maturation is neither frequent nor required for genome survival. This type of chronic intracellular infection without mature virus production can allow accumulation of mutations in genome segments involved in maturation of virus that might otherwise be nonviable or noncompetitive, as discussed earlier for SSPE. Finally, with a variety of animal RNA viruses the presence of DI particles selects for infectious virus mutants (Sdi^- mutants) that are relatively resistant to DI particle interference (76, 82, 83, 86). The repetitive escape of Sdi^- mutants from DI particle interference followed by recurring generation of new DI particle types can drive rapid viral genome evolution (77).

SUMMARY

The high error rate inherent in all RNA synthesis provides RNA virus genomes with extremely high mutation rates. Thus nearly all large RNA virus clonal populations are quasispecies collections of differing, related genomes (14, 49). These rapidly mutating populations can remain remarkably stable under certain conditions of replication. Under other conditions, virus-

population equilibria become disturbed, and extremely rapid evolution can result. This extreme variability and rapid evolution can cause severe problems with previously unknown virus diseases (such as AIDS). It also presents daunting challenges for the design of effective vaccines for the control of diseases caused by rapidly evolving RNA virus populations.

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