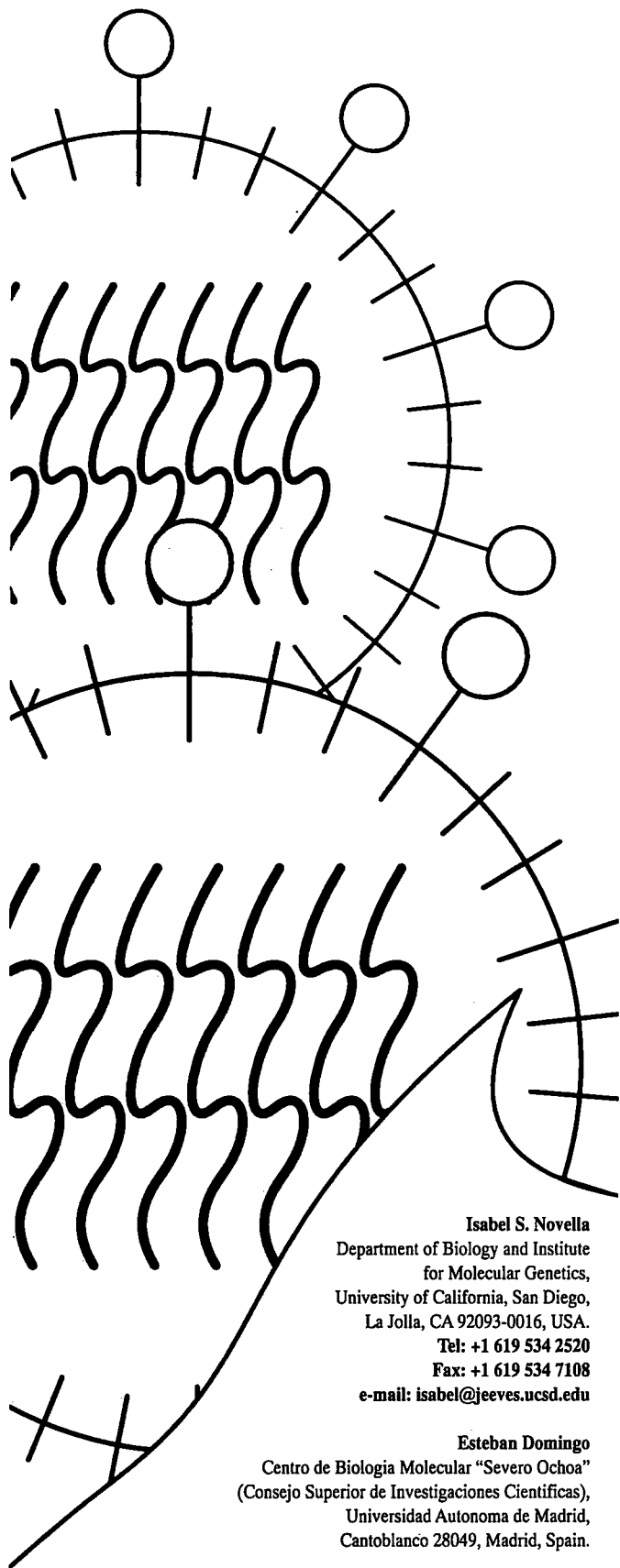




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Rapid viral quasispecies evolution: implications for vaccine and drug strategies

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High mutation rates occurring during replication allow RNA viruses to evolve rapidly and adapt continuously to new environments. This poses an enormous challenge to vaccine and drug development which, to be effective, must consider RNA virus variability and follow approaches that minimize the probability of escape or resistant mutants arising.

RNA viruses are responsible for a large number of human diseases such as polio, influenza, rabies and many others, including 'newly emerging' infections such as hantavirus hemorrhagic fever and Ebola (see Table 1).

The evolution of RNA viruses is strongly determined by remarkable variability. Extremely high mutation rates (10^{-3} – 10^{-5} incorporations per base copied)² and both homologous and nonhomologous recombination allow extensive generation of new sequences. Most mutations will be deleterious and eliminated by natural selection, but many have selective value in the particular environment which the virus is replicating, and viruses harboring such mutations may become dominant in the population. The continuous generation of variants during replication, independent of their selective value, produces a complex distribution of nonidentical genomes: the quasispecies⁴.

Many factors can influence the long-term evolution of RNA viruses, one of which is population size. Thus, darwinian selection is usually the driving force during transmission of large virus populations; during replication of viral quasispecies⁵, whereas random drift

Table 1. Some RNA viruses that are important human pathogens^a

Family of virus	Virus name	Human disease
Picornavirus	Poliovirus	Polio
	Coxsackie virus	Meningitis
	Echovirus	Meningitis
	Enterovirus	Meningitis
	Rhinovirus	Common cold
	Hepatitis A virus	Infectious hepatitis
Calicivirus	Norwalk-like viruses	Gastroenteritis
		Gastroenteritis
Astrovirus	Human astroviruses	Gastroenteritis
Togavirus	Eastern encephalitis	Fever, encephalitis
	Western encephalitis	
	Venezuelan encephalitis	
Flavivirus	Rubella	German measles
	Hepatitis C	Post-transfusion hepatitis
	Yellow fever	Yellow fever
	Dengue	Dengue fever
	Japanese encephalitis	Encephalitis
Corona virus	Human enteric coronaviruses	Coronavirus cold
	Coronavirus	
Paramyxovirus	Human parainfluenza	Pneumonia, bronchiolitis, upper respiratory tract infection, croup
	Mumps virus	Mumps
	Measles virus	Measles, subacute sclerosing panencephalitis
Pneumovirus	Respiratory syncytial virus	Bronchiolitis, pneumonia
Rhabdovirus	Vesicular stomatitis	Virus fever, stomatitis
	Rabies virus	Rabies
Filovirus	Ebola virus	Hemorrhagic fever
	Marburg virus	Hemorrhagic fever
	Reston virus	Hemorrhagic fever (in primates)
Orthomyxovirus	Influenza virus	Influenza
Arenavirus	Lassa virus	Lassa hemorrhagic fever
	South America arenaviruses (Junin, Machupo)	Hemorrhagic fever
Bunyavirus	Rift Valley fever virus	Hemorrhagic fever
	Sandfly fever	Sandfly fever
	La Crosse	California encephalitis
	Crimean Congo fever	Hemorrhagic fever
Hantavirus	Hantaan virus	Hemorrhagic fever, nephropathy, respiratory distress
	(Four Corners/Muerto Canyon/Sin Nombre)	
Reovirus	Rotavirus	Infantile diarrhea
Retrovirus	HTLV-1	Adult T-cell leukemia
		Tropical spastic paraparesis
	HIV-1	AIDS
	HIV-2	AIDS

^aData from Ref. 1.

Glossary:

Cryptic immunopathological responses – Hidden/subclinical tissue damage resulting from an inappropriate immune response to the pathogen.

Defective interfering particle – Virus particles produced during an infectious cycle that are not able to replicate themselves.

Escape mutants – Viruses that are resistant to the effect of drug or antibody as a result of sequence changes within the site of action of the antiviral agent.

Gamma-globulin prophylaxis – Administration of antibodies to prevent infection.

Homologous/non-homologous recombination – Transfer of genetic information from one genome or chromosome to another during DNA replication which is initiated by the pairing of complementary/non-complementary sequences within the genome.

Live-attenuated virus vaccine – Vaccine based upon a replication-competent virus that has been crippled or weakened (usually by empirical methods such as repeated passage in tissue culture or the non-natural host) to reduce its pathological effects.

Plantigens and plantibodies – Viral proteins or antiviral antibodies produced in genetically modified plants.

Random drift – Gradual accumulation of mutations within the genome.

Replicons – Unit of nucleic acid that contains all the sequence necessary for replication.

Subacute sclerosing panencephalitis – A rare fatal disease of children or adolescents years after measles infection, resulting from slow replication of defective measles virus within the brain.

Tautomers – A compound that differs from its isomer, with which it readily interconverts, only in the positions of a mobile atom, frequently hydrogen, and of double bonds.

Virus reassortants – Novel progeny virus formed by random association and packaging of genome segments during mixed infection of a cell by different RNA virus species.

prevalent during genetic bottlenecks^{6,7}. Natural selection is extremely effective. Extensive genetic heterogeneity has often been found not only among infected individuals during an outbreak, but also within single hosts or even particular tissues. Selection of drug-resistant mutants *in vivo* during treatment strongly suggests that specific adaptation does occur rapidly, as has been shown in cell culture⁸. Obviously, evolution will depend on the environmental conditions under which viral populations replicate. These environmental conditions include, among others, the immune response of the host, antiviral intervention, fever and hormone status. Consequently, effective drug and vaccine development should focus on approaches that induce a broad variety of defenses, so that the probability of generating mutants

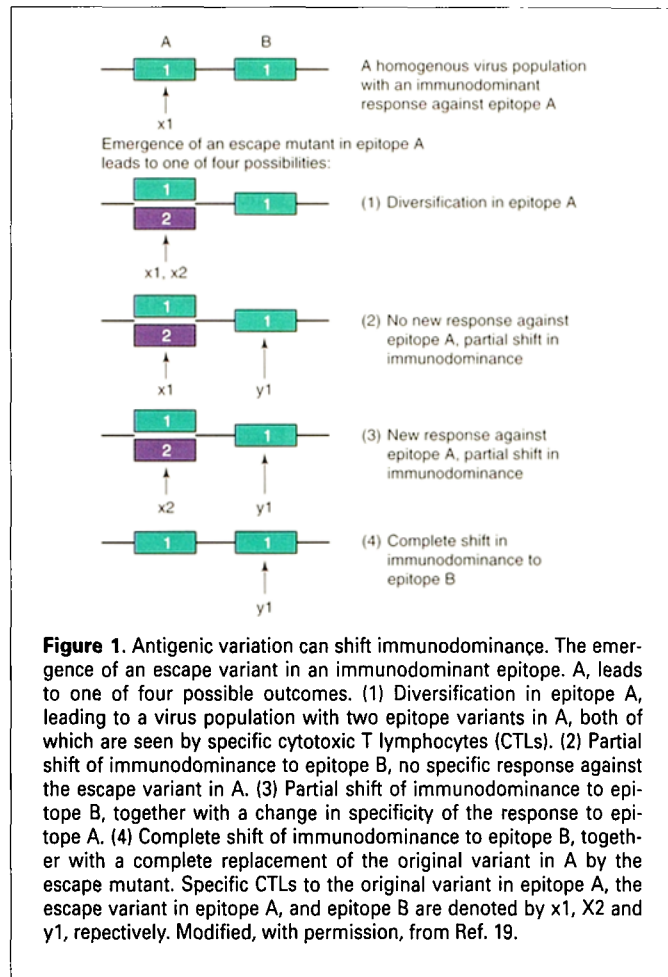


Figure 1. Antigenic variation can shift immunodominance. The emergence of an escape variant in an immunodominant epitope, A, leads to one of four possible outcomes. (1) Diversification in epitope A, leading to a virus population with two epitope variants in A, both of which are seen by specific cytotoxic T lymphocytes (CTLs). (2) Partial shift of immunodominance to epitope B, no specific response against the escape variant in A. (3) Partial shift of immunodominance to epitope B, together with a change in specificity of the response to epitope A. (4) Complete shift of immunodominance to epitope B, together with a complete replacement of the original variant in A by the escape mutant. Specific CTLs to the original variant in epitope A, the escape variant in epitope A, and epitope B are denoted by x1, x2 and y1, respectively. Modified, with permission, from Ref. 19.

that can escape all of them (i.e. multiple mutations in a single genome) is minimized.

Implications for vaccine design

For vaccines, formulations based on single or a few viral peptide sequences (i.e. a limited number of epitopes) often induce good responses against the homologous sequence, and usually have the advantages of safety and stability. However, achievement of adequate levels of protection in a population will be problematic. Some vaccine trials of HIV and foot and mouth disease virus (FMDV) are showing this problem^{9,10}. As a result of the changing and unpredictable dynamics of RNA virus quasispecies, these vaccines may not always prevent infection by related virus isolates, nor prevent selection of escape mutants during primary infection¹¹. Peptide vaccines for RNA viruses have sometimes been successful when challenge or reactivity tests were performed with homologous strains, but results with heterologous strains have frequently given disappointing results. Examples are HIV (Ref. 12) and hepatitis B virus¹³.

No peptide vaccine is currently licensed to prevent diseases caused by RNA viruses. Subunit vaccines (i.e. vaccines consisting of viral proteins) have the same kinds of problem as peptide vaccines, and they are often poorly immunogenic. Efforts focusing on the use of a cloned HIV envelope glycoprotein in a vaccine for AIDS have faced this problem and other approaches must be examined¹⁴.

Additional concerns are stability and the possibility of denaturation during preparation, and the induction of an abnormal immune response leading to enhancement of disease after infection. This has been described for respiratory syncytial virus (RSV) (Ref. 15). One subunit vaccine that has been rather successful is the vaccine for feline leukemia virus (FeLV), but escape mutants have already been reported¹⁶.

The problem of viable escape mutants is also influenced by factors other than mutation rates. Viruses that replicate very rapidly will be more likely to generate and select such mutants during an infection although the immune response clears many millions of infectious particles per day. This occurs with HIV (Refs 17,18). In addition, viruses that can cause immunosuppression or establish persistent infection (particularly when they can avoid displaying viral antigens at the cell surface), such as lymphocytic choriomeningitis virus, are difficult to control. Nevertheless, the most important factor that will affect the generation of viable escape mutants is the nature of the virus itself. Thus, different viruses show different antigenic epitopes, and each epitope may or may not tolerate extensive substitutions. Substitutions that confer antibody resistance to some conserved epitopes will be deleterious and escape mutants will be unable to survive selection. This explains the lack of escape mutants for successful RNA virus vaccines such as those for poliovirus, measles and mumps. Conversely, the more variable and adaptable antigenic epitopes in viruses such as FMDV and HIV-1 pose much greater challenges (Fig. 1).

At the other end of the spectrum, live-attenuated virus vaccines inevitably include not only an ample repertoire of B-cell and T-cell epitopes, but also a broad spectrum of mutations, because they consist of diverse quasispecies populations. As attenuation may limit, but not prevent, viral replication, the number of sequences that the immune system encounters becomes even more extensive. Most of the successful vaccines against RNA (and DNA) viruses have been live-attenuated viruses, including those used to control poliomyelitis, smallpox, measles, rubella and mumps. Promising results have also been reported for RSV and parainfluenza virus¹⁵. However, the use of these vaccines inevitably involves some risks. Use of live RNA virus always allows some possibility for virulent revertants to arise by mutation and/or by recombination of attenuated precursors, a phenomenon described for poliovirus²⁰. This problem might be circumvented by developing strains with a very high detrimental mutational load distributed along the genome and, of course, by in-depth knowledge of the factors that contribute to attenuation in vaccinees.

The selection of viral strains to develop a vaccine is a critical, difficult and uncertain enterprise. Cloning of virus from natural isolates, or cell-culture replication of such isolates in the laboratory, is likely to select for sequences that are different from those in circulating strains, either because of random sampling or natural selection (see above). This seems to be happening with HIV vaccines¹². During the preparation of vaccine batches, the enormous ability of RNA viruses

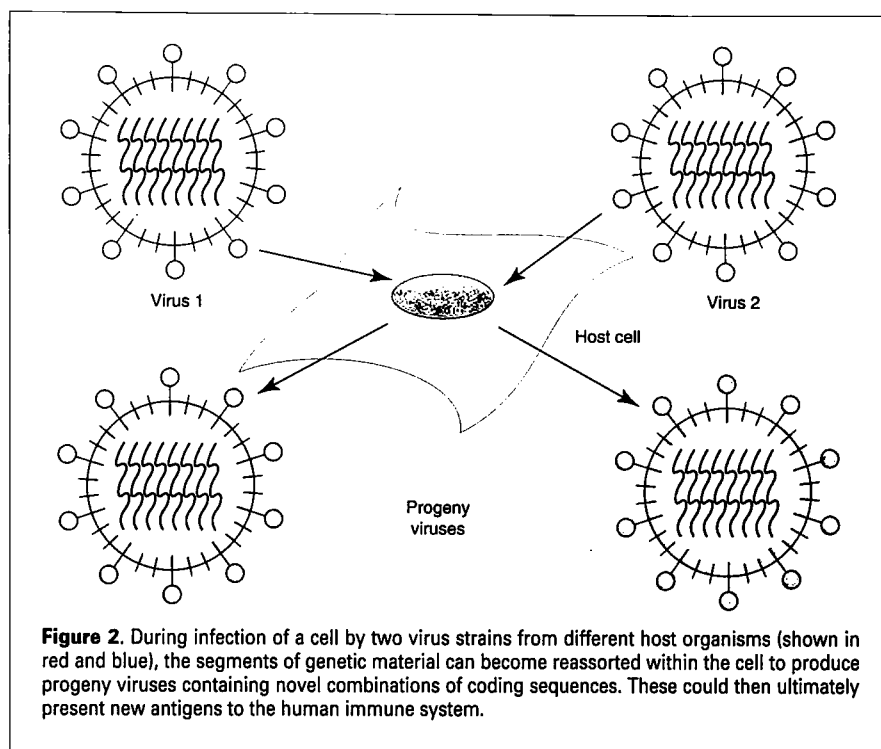


Figure 2. During infection of a cell by two virus strains from different host organisms (shown in red and blue), the segments of genetic material can become reassorted within the cell to produce progeny viruses containing novel combinations of coding sequences. These could then ultimately present new antigens to the human immune system.

to recover fitness (and possibly virulence) during massive replication⁵ must always be kept in mind. Studies of vaccine safety must in addition include studies of differences among age/sex/ethnic groups of vaccinees. Simian immunodeficiency virus deletion mutant vaccines developed as a model for HIV (Ref. 21) seem to be safe for adult monkeys, but Ruprecht and co-workers have found that they are not safe for newborn monkeys after mucosal infection²². Another potential problem is the rare mutational generation of genomes that can produce atypical infections or cryptic immunopathological responses, an effect observed for several viruses, including defective measles viruses, causing subacute sclerosing panencephalitis²³. Finally, the potential for inapparent (but damaging) long-term persistent infections and/or proviral DNA integration and host-cell chromosome alterations must be considered. Live vaccines for HIV or hepatitis C virus might have this potential.

Some successful vaccination programs will lead to virus eradication but others may leave a fraction of the population susceptible or cryptically infected. For certain viruses, the existence of animal reservoirs can provide a continuous source of infection. Under these circumstances, RNA viruses replicating in susceptible hosts (or vectors) could occasionally generate new antibody-escape variants that can infect a previously immune population. A very well-known example of such behavior is the rare production of influenza A virus reassortants (Fig. 2) carrying segments from fowl and human influenza genomes. These include new epitopes that are not recognized by previously induced immune responses in the human population and they initiate human pandemics. This might also occur with a number of other RNA viruses²⁴. Thus, during disease outbreaks, considerable effort must focus on the isolation and characterization of evolving strains involved in the disease process, and vaccine producers should be prepared to replace formulations that may have become less effective¹⁰. This is acknowledged by the annual review of prevalent viral subtypes

included in vaccines against influenza. These need to take into account changes resulting from hypervariability in some epitopes (Fig. 3).

Problems for effectiveness of antiviral agents

RNA virus variation is a constant hurdle for the development of effective antiviral drugs. As for vaccine design, drug therapies must deal with the rapid appearance of resistant virus mutants, but, with

antiviral drugs, the problem is aggravated. Usually, adequate vaccination will induce an immune response against a variety of epitopes and different variants of each epitope, so that escape mutants must necessarily include multiple appropriate, non-debilitating mutations. Accordingly, their appearance is much less probable. However, most antiviral drugs exert their functions on protein domains that usually involve a very limited number of residues (for example, the catalytic site or contiguous sites of enzymes). The direct consequence is that resistant viruses are often single point mutants, and the probability for rapid takeover by such mutants is high. The use of monoclonal antibodies in the treatment of RNA virus infections is impaired for the same reason. There are several examples in the literature of resistant mutants being isolated after only weeks or even one or two days of antiviral treatment²⁴. Furthermore, such resistant variants can be present and represent a significant fraction of the quasispecies population even before treatment²⁶.

One way to decelerate rapid generation of escape mutants might be to use combination therapies directed toward different steps of the virus life cycle. This might be achieved by the use of antiviral drug cocktails or by the use of drugs with multiple targets. The anti-HIV ribozyme approach developed by Wong-Staal and colleagues²⁷ might provide one such antiviral therapy if a number of target sequences can be included. Another novel approach might involve attempts toward specific manipulation of RNA virus mutation rates. These are normally within the narrow range that provides optimal biological adaptability (near to the error threshold). Much lower mutation rates could minimize the appearance of mutants resistant to other antivirals, while higher mutation rates would cause the quasispecies to exceed the error threshold and to lose meaningful genetic information (mutational meltdown)⁴. Eventually, rational drug design approaches based on knowledge of the structure and function of viral polymerase domains might allow the development of virus-specific mutagenic drugs. Viral kinases are another potential target for design of specific ribonucleotide analogs that can be efficiently phosphorylated only in virus-infected cells. The first widely employed antiviral compound, iododeoxyuridine, was developed in the late 1950s. It is mutagenic due to increased formation of rare tautomers, and abundant viral kinases promote its preferential phosphorylation within herpesvirus-infected cells²⁸. As we enter the next millenium, specific drug-design approaches will surely be capable of producing much more specific antiviral mutagens.

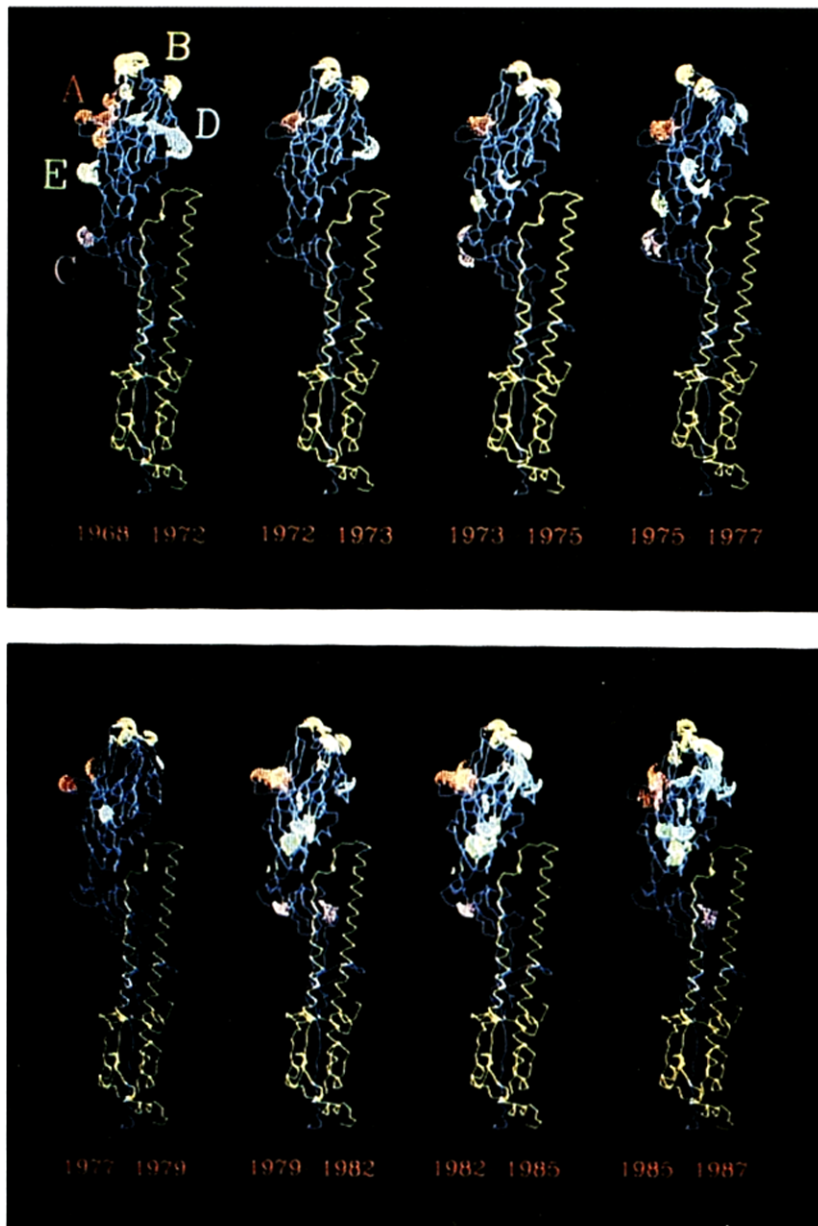


Figure 3. Natural variation in the Ha of H3 strains from 1968–1987. (a) Location of amino acid differences shown on the HA monomer in strains from years of significant influenza activity are shown by solvent accessible surfaces to represent the altered HA antigenic surface: site A, red; site B, yellow; site C, magenta; site D, blue; and site E, green. Generally, when changes occur throughout antigenic sites A–E, a new epidemic strain emerges. (Reproduced, with permission, from Ref. 25.)

Conclusions

Vaccination campaigns and antiviral drug therapy are new selective forces shaping viral evolution. Although relative success has been achieved, RNA viruses have shown an immense ability to respond to such changes in selective pressures. Many other relevant selective changes are rapidly occurring in our biosphere, largely due to human activities and human population growth. This not only affects the evolution of viral quasispecies in natural hosts, but also provides new opportunities for RNA viruses to expand their diverse quasispecies populations, or even to jump to new niches and new hosts. Novel virus diseases are emerging and more will emerge in the future, as some old ones disappear (and sometimes reappear). Any attempt to control and eradicate RNA viruses must deal with the fact of extreme variation and rapid evolution. The better we understand RNA viruses as complex quasispecies populations, the closer we will come to effective measures for their control.

The outstanding questions

- Can vaccine protection against RNA viruses be provided by direct intramuscular injection of DNA preparations engineered to produce highly mutagenized mRNA (and diverse proteins)?
- Can intestinal colonization with engineered avirulent bacterial vectors expressing multiple viral antigens provide effective vaccination?
- Can multiple diverse viral proteins, expressed in dietary vegetables (plantigens) provide protection against intestinal (and other) RNA viruses? Can plantibodies?
- Can improved technology with human hybridomas eventually produce cost-effective, concentrated, highly diverse polyclonal antiviral antibody preparations for use in Gamma-globulin prophylaxis and/or treatment?
- Can we use the principles of interference by defective interfering particles for the design of antiviral replicons which replicate and interfere only during virus infections?
- How will RNA virus evolution be affected by widespread employment of many new vaccines, antiviral drugs and other antiviral therapies?
- How can we employ diversity to combat the problems of RNA virus diversity?

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