Genetic Variability: The Key Problem in the Prevention and Therapy of RNA-Based Virus Infections

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Abstract: Despite extraordinary progress that has recently been made in biomedical sciences, viral infectious diseases still remain one of the most serious world health problems. Among the different types of viruses, those using RNA as their genetic material (RNA viruses and retroviruses) are especially dangerous. At present there is no medicine allowing an effective treatment of RNA-based virus infections. Many RNA viruses and retroviruses need only a few weeks to escape immune response or to produce drug-resistant mutants. This seems to be the obvious consequence of the unusual genetic variability of RNA-based viruses. An individual virus does not form a homogenous population but rather a set of similar but not identical variants. In consequence, RNA-based viruses can easily adapt to environmental changes, also those resulting from immune system response or therapy. The modifications identified within viral genes can be divided into two groups: point mutations and complex genome rearrangements. The former arises mainly during error-prone replication, whereas RNA recombination and generic reassortment are responsible for the latter. This article shortly describes major strategies used to control virus infections. Then, it presents the various mechanisms generating the genetic diversity of RNA-based viruses, which are most probably the main cause of clinical problems.
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Key words: RNA-based viruses; viral infections—prevention and therapy; genetic variability; error-prone replication; RNA recombination; genetic reassortment

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1. INTRODUCTION

Theoretically, a virus has only one chance during all our lifetime to attack us successfully.[1–3] Initially, the virus tries to overcome non-specific immune barriers, i.e., innate immunity, which constitutes the first line of defense against infections. It includes the barriers of the skin and mucous membranes, enzymes in secretions, and phagocytic cells. Then, a virus recognizes suitable receptors located on the surface of sensitive cells and penetrates inside. Using proteins encoded by itself, as well as numerous host proteins, the virus replicates, and finally leaves the cells. As long as the virus stays inside the cell, the development of infection is inhibited owing to cell-mediated immunity. It is based on the presence of activated T lymphocytes (acting as cytotoxic cells) and on releasing lymphokines, which activate monocytes and macrophages. Several days after the moment of infection, a humoral immunity is also activated and virus-specific antibodies are generated. If the immune system works effectively, the virus is eliminated. Because of the presence of specific antibodies the organism is already protected from a reinfection or a next infection by the same microorganisms.[1–4]

In practice, the development and consequences of virus infection depend on many factors, among others, on the virus' dose and its biological properties, on the way of infection, on the virus' effect on cell physiological functions, as well as on the fitness of the immune system.[1–4] As a result, viral infection can be local or general, acute or chronic, symptomatic or asymptomatic.

In spite of rapid development of medical sciences, viral diseases continue to be one of the major world health problems, being the most frequent cause of human morbidity and mortality on the world scale.[1] Among different types of viruses, those containing RNA as their genetic material are especially difficult to combat. Several lines of evidence suggest that RNA-based viruses can easily produce drug-resistant mutants or evade host immune response mainly thanks to their unique ability to change rapidly and adapt to new situations.[5–10] Accordingly, this review shortly presents currently available methods of prevention and therapy of viral infections and then it concentrates on the different processes generating the enormous genetic plasticity of RNA-based viruses.

2. PREVENTION AND THERAPY OF VIRAL INFECTIONS

Together with a continuous increase of our knowledge on viruses and relations between the virus and the infected organism, new methods of fighting viral infections are continually being developed. Generally, they can be divided into three basic categories: immunization, chemotherapy, and immunomodulation.

A. Immunization

Immunization is based on the natural abilities of the organism to defend itself against an infection.[1–3] As a result of immunization, specific antibodies are introduced into or generated in the organism to protect it against the invasion of a pathogenic agent. Depending on how antibodies appear in the organism, immunization is called passive or active. Passive immunization is performed by direct administration (intravenous or intramuscular injection) of exogenous antibodies.[11] Active immunization (vaccination) induces the formation of endogenous antibodies by administration of a suitably prepared virus or its fragment.[4]

1. Passive Immunization

It consists in the usage of antibodies (collected from an immune individual) to protect an infected, non-immune individual.[11,12] The most frequently applied preparation is a protein fraction from human blood plasma. It contains the majority of antibodies present in the blood of a healthy adult person. The administration of immunoglobulins supports the humoral type of immunity and can,

therefore, be effective only when a virus has not yet penetrated the cell. In clinical practice, that kind of immunization is used in the prophylactics of some infectious diseases (e.g., if an infection with rabies virus is suspected) or in patients with especially severe symptoms or complications (i.e., immunodeficiency).[11]

There are several reasons why passive immunization is not commonly used, although it may immediately slow down or inhibit viral infection.[11–14] Exogenous immunoglobulins retain their activity only for a short time. Their administration creates the risk of serious side effects, for instance, an allergic reaction to foreign proteins or the risk of transmitting another infectious agent. Moreover, it is not easy to obtain suitable preparations satisfying pharmaceutical requirements and in consequence the costs of their application are very high. Normal pooled human immunoglobulin (500 mg dose) contains enough antibodies against common infections to protect a patient for 3–4 weeks. However, over 1,000 donors are used to prepare one dose.[12]

2. Active Immunization—Vaccination

The history of vaccination was initiated hundreds of years ago. The Roman scientist Plinius the Elder (lived 23–79 A.D.) proved that the livers of dogs suffering from rabies contain a remedy protecting against this disease. In the XVIth century, Asian doctors made children immune to smallpox by rubbing scabs taken from sick persons into the children's skin. A real breakthrough occurred in the year 1796, when the British doctor Edward Jenner found that the cowpox virus can be used for effective and safe vaccination of humans.

However, it is only at the beginning of this century that the techniques of vaccine production were significantly improved owing to the development of efficient methods of microorganism propagation and isolation. That has resulted in obtaining many new antiviral vaccines enabling preventive immunization. The greatest successes associated with the measures undertaken at that time were smallpox eradication[15] and a considerable limitation of poliomyelitis.[16] The first vaccines contained a small number of attenuated viruses and were traditionally administrated by injection, with the exception of the oral Sabin vaccine against poliovirus.[16] However, the techniques available at that time did not permit to obtain vaccines against all pathogenic viruses, mainly because of difficulties associated with the production of attenuated viruses. The achievements made in medicine and biology during the last 20 years have markedly revived this field of scientific research bringing hopes that infectious diseases will be significantly limited.

Antiviral vaccines produced at present can be divided into four basic groups, i.e., vaccines containing live viruses, vaccines containing inactive viruses or their fragments, vaccines containing so-called anti-idiotype antibodies, and DNA vaccines[17] (Fig. 1).

The already mentioned vaccines obtained by Jenner or by Sabin as well as vaccines against measles, mumps, and rubella belong to the first group. To obtain these vaccines a suitable method of virus propagation and attenuation is required. The main danger involved in the application of vaccines containing attenuated, but living viruses, are postvaccination infections.[16] They may occur both in people subjected to vaccination as well as in persons being in contact with the immunized patient. The second group is represented by vaccines containing inactivated viruses or their fragments, for instance Salk intramuscular vaccine against poliovirus,[18,19] vaccines against influenza virus,[20] or vaccines against hepatitis A or hepatitis B virus.[21–23] The role of immunogenic agents in them is played by various particles. The most frequently used are: killed viruses devoid of biological activity,[18,19] virus-like particles (e.g., empty capsids),[24,25] split viral particles, or individual viral proteins.[21–23] Owing to the use of modern biotechnological methods, natural viral proteins are substituted more and more frequently by analogues produced in cell cultures (of both, prokaryotic[26–28] and eukaryotic cells[29–31]), in transgenic plants[32] or by chemical synthesis.[33,34]

The next group is constituted by anti-idiotype vaccines containing anti-idiotype antibodies. The strategy used to obtain them is presented in Figure 2. It is known that active immunization or infection

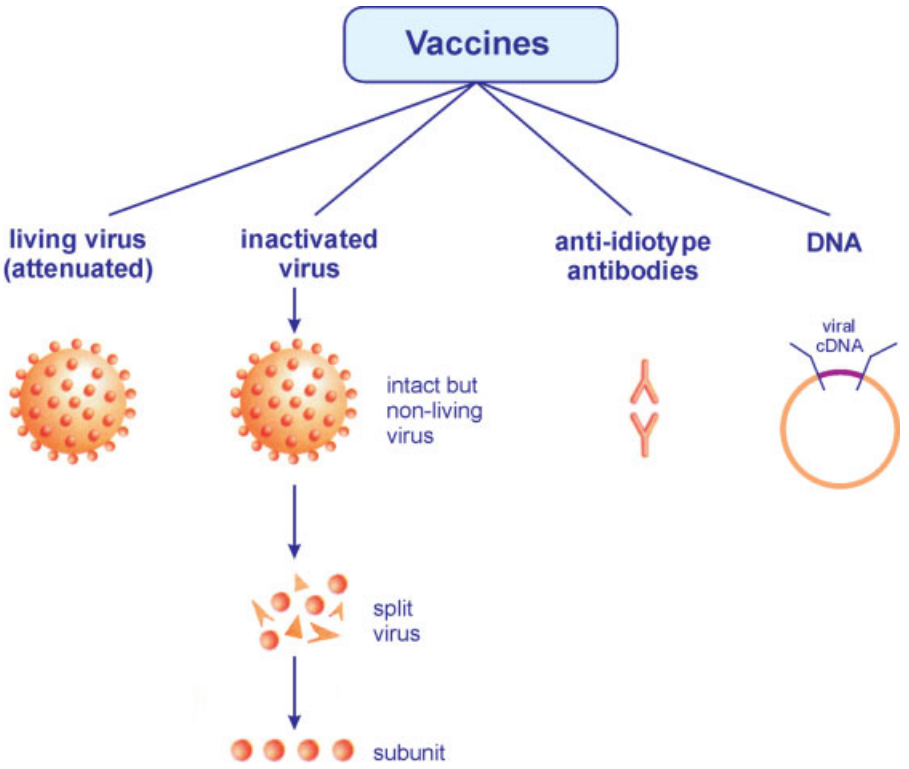

Figure 1. Currently used or tested antiviral vaccines. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

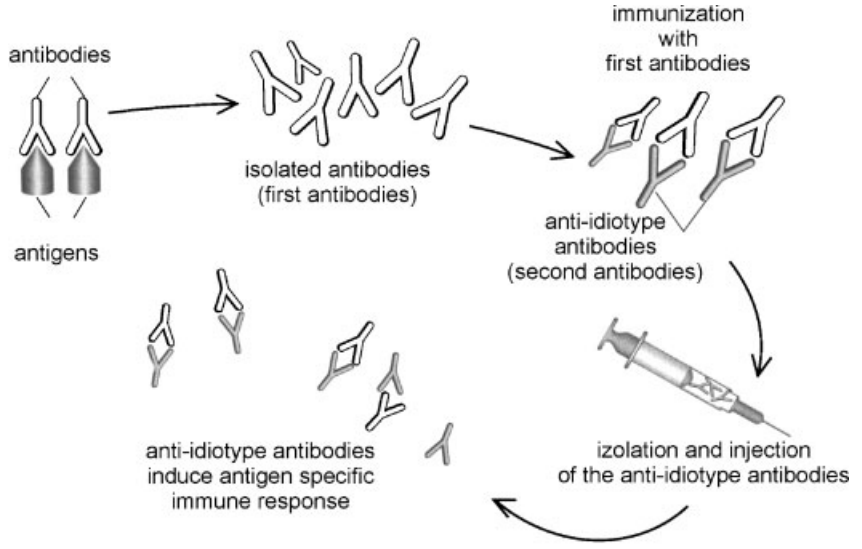

Figure 2. Anti-idiotype vaccine preparation. To obtain an anti-idiotype vaccine, antibodies characteristic for the given antigen (first antibodies—in white) are isolated, purified, and afterwards used as an immunizing factor. As a result, so-called anti-idiotype antibodies are generated (second antibodies—in gray). Their variable fragment possesses a structure analogous to that of an antigen. Anti-idiotype antibodies can therefore be used as a vaccine inducing the formation of antibodies specific to the antigen.

cause the production of antibodies (first antibodies). Their variable fragment abbreviated Fab is responsible for antigen binding. The Fab region directly engaged in interaction with the antigen is called an idiotype. It represents a reverse image, a kind of negative of the attached protein. If, therefore, the first antibodies are used as immunizing agents, then the idiotypes of newly generated antibodies (second antibodies) should have an identical or similar structure as an antigen. So, secondary antibodies may be used as a vaccine inducing immune response against a specific viral antigen. This approach permitted to induce an immune response against reoviruses, poliovirus, rabies virus, Coxsackie virus, or hepatitis B virus.[35–38] The observed level of antibodies was, however, noticeably lower than when inactivated viruses or even single viral proteins were used.

Studies conducted in the last few years have shown that DNA can also be applied as a vaccine (Fig. 3).[39,40] It appears that genes isolated from pathogenic microorganisms can be introduced into the cells of another organism through recombination. DNA vaccines supply cells with foreign genetic material, that is, with DNA encoding viral protein accompanied by adequately selected regulatory DNA sequences, which ensure an effective expression of the antigen. The presence of a viral protein in the cells of a transformed organism stimulates an immune response.

The initial successes associated with the obtaining and introduction of vaccines suggested that they should be effective in the case of each pathogenic microorganism. Recent experience gained during the studies on human immunodeficiency virus (HIV), hepatitis C virus (HCV), Ebola virus, and other viruses have forced us to verify this opinion. Specific properties of some viruses, may cause the immune response induced by a vaccine to be completely ineffective.

B. Chemotherapy

Problems connected with vaccine preparation have inclined investigators to search for other methods of fighting virus infections. Then, an idea arose to use relatively simple chemical compounds to inhibit fundamental life functions of viruses.[41–43] Initially, a search for such compounds was conducted by trial and error. However, with the increase of available data on the structure and properties of viral proteins, a method of drug design has been created. Studies conducted nowadays are most frequently aimed at finding some chemical compounds inhibiting the activity of viral polymerases[41,42,44,45] and proteases.[41,42,46,47]

All RNA viruses and many DNA viruses encode specific polymerases. They are responsible for the selective replication of viral genomic molecules. The so-far obtained drugs disturbing the functioning of viral polymerases can be divided into two main groups: inhibitors of DNA or RNA synthesis (RNA/DNA synthesis terminators)[41,42,44,48] and inhibitors of polymerases.[41,42,45] The first group comprises nucleoside derivatives (for instance, AZT or acyklovir) terminating DNA/RNA synthesis (all of them lack 3′-OH moieties). Polymerase recognizes them as a substrate (a normal nucleotide) and incorporates them into a newly synthesized nascent strand. Their structure, however, makes a further elongation of DNA/RNA, (i.e., the addition of the next nucleotides by 3′–5′ phosphodiester bond formation) impossible.[41,42,44,48]

The second group is constituted by compounds which are not nucleoside derivatives. As a result viral polymerase inhibitors are not substrates in the reaction of nucleic acid polymerization. They are projected in such a way as to make them able to bind specifically to polymerase catalytic center or in its nearest proximity and thereby to inhibit enzyme activity (e.g., nevirapine, efavirenz).[41,42,45]

Another target of antiviral therapy are viral proteases. Their action is absolutely indispensable when the virus encodes only one large polyprotein which is transformed into active proteins through several specific cleavages. The latter process is catalyzed by virus and host encoded proteases. In the last few years, many compounds effectively blocking the activity of viral proteases and particularly HIV protease (e.g., saquinavir, nelfinavir)[41,42,46] have been identified.

Recently, a new approach called the drug selection method has become a certain alternative for the earlier mentioned drug design method. Among others, the so-called oligoribonucleotide

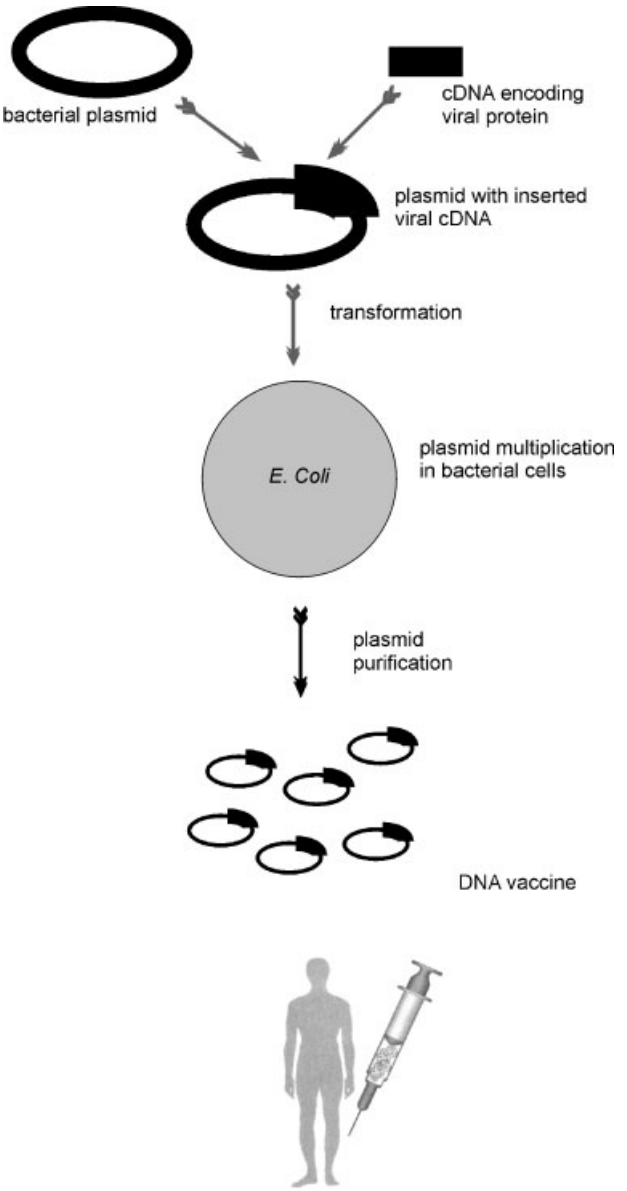

Figure 3. Preparation of the DNA vaccine. The procedure allowing the acquiring of DNA vaccine is relatively simple. The chosen viral gene is cloned and then introduced into the bacterial plasmid, along with a suitable promoter sequence enabling the expression of the viral gene in host cells. The resultant plasmid is multiplied in bacteria (e.g., in *E. coli*), isolated, and purified. In this form it can be used as a vaccine.

inhibitors of polymerases were obtained in this way.[49] Viral polymerases are known to replicate selectively their own genomic molecules thanks to the ability to recognize and bind them specifically. Thus, the *in vitro* selection method (SELEX—systematic evolution of ligands by exponential enrichment)[50] has been used to obtain short oligonucleotides selectively binding viral replicases and preventing the initiation of a replication process.[49] The use of the *in vitro* selection method also makes it possible to obtain small RNA molecules called ribozymes, which selectively cleave viral genomic RNAs.[51–55]

C. Immunomodulation

Immunomodulation may be defined as an action aimed at the activation of the immune system to enhance organism response to infection. Its application in antiviral therapy has become possible after the discovery of cytokines.[56] These are small polypeptides or glycoproteins (their mass is usually lower than 30 kDa) playing the role of intracellular signaling molecules.[56,57] The members of the cytokine family are very diversified and show a certain similarity to hormones or growth factors.[58–61] From a virological standpoint, cytokines inducing or assisting the action of the immune system are especially important.[62] The best studied, and practically used representatives of this group are interferons.[62–64] At present, two basic classes are distinguished among them: interferon of type I (IEN-α/β) and interferon of type II (IEN-γ). In addition, IFNα/β may be divided into four subgroups: IFN-α, IFN-β, IFN-ω, and IFN-τ, and each of them undergoes specific expression. In a human organism, a viral infection induces the production of IFN-β by non-hematopoietic cells, and IFN-α, IFN-ω, and various amounts of IFN-β by hematopoietic cells.[62,65]

D. Clinical Practice

The information presented above may indicate that there are many potential means and ways enabling effective control of viral infections. Unfortunately, a considerable part of them have not found practical application and only some of the newly discovered antiviral vaccines or drugs have been used in clinical tests. What, therefore, do doctors have at their disposal if they want to protect someone against viral infection or subject him/her to the antiviral therapy?

At present, the main weapon in our combat with viral infections is prophylaxis, i.e., preventive vaccination (Table I) and the continuous screening of persons belonging to groups of special risk. Consequently, we have less and less problems with diseases such as poliomyelitis, measles, rubella, mumps, hepatitis A and hepatitis B, or influenza in the case of which the preventive vaccination is compulsory or effective vaccines are available. However, there is still a long list of viruses, for which no effective vaccines have been obtained. This also concerns those viruses whose genomic molecules were cloned and every viral protein can easily be produced in cell culture. Theoretically, such an antigen should induce immune response, which protects against infection. However, for some reasons it does not happen.[66,67]

The situation seems to be even worse if we look at the list of antiviral drugs, which are currently available (Table II). The presented data indicate that effective treatment is possible only in the case of infections induced by some DNA viruses. Because of particularly intensive studies on HIV conducted in the 1990s, several drugs inhibiting HIV development were also found.[42] However, clinical practice showed that none of them is sufficiently effective. Usually HIV requires merely several weeks or months to produce a strain resistant to the applied medicine.[42,68] As a result, the only chance to increase the effectiveness of AIDS treatment is a simultaneous application of several pharmacologicals. Similarly poor effects are observed during HCV infection treatment with interferon-α and ribavirin. Although patients are carefully selected for the therapy, only 40% of them eliminate the virus.[69]

This raises a very intriguing question of why the prevention and treatment of virus infections are so difficult especially if their genome is composed of RNA molecules. Studies conducted during the last decades brought many different answers. It was demonstrated that DNA viruses, particularly those heaving large genomes (encoding ca. 200 proteins) have developed very sophisticated methods of evading immune defense.[70] Adenoviruses or herpesviruses encode proteins which are able to reduce the expression of MHC class I antigens on host cells. As a consequence, T-cells cannot recognize infected cells.[71–74] Adenoviruses, Kaposi sarcoma herpesvirus, Epstein–Barr virus can also suppress MHC class II antigens.[70,73,75,76] There are also DNA viruses producing homologues of cytokine receptors or cytokines, which interfere with the host's immune system.[70,77,78] A very interesting defense against the actions of interferons was developed by adenoviruses and

Table I. Widely Available and Currently Tested Antiviral Vaccines

Virus	*Viral genome*	*Type of vaccine*
Poliovirus	+RNA	Types 1, 2, 3 (oral)
		Killed whole viruses
Measles	−RNA	Live attenuated viruses
Mumps	−RNA	Live attenuated viruses
Rubella	+RNA	Live attenuated viruses
Yellow fever	+RNA	Live attenuated viruses
Varicella-zoster	ds DNA	Live attenuated viruses
Hepatitis A	+RNA	Killed whole viruses
		Live attenuated viruses[a]
Rabies	−RNA	Killed whole viruses
Influenza	−RNA	Killed whole viruses
		Split viruses
		Subunit of viruses
		Live attenuated viruses[a]
Hepatitis B	Semicircular ds DNA	Subunit of viruses
Vaccinia	ds DNA	Live attenuated viruses
Rotavirus	ds RNA	Live attenuated viruses[b]
Adenovirus	ds DNA	Live attenuated viruses[c]
Tick-borne encephalitis virus	+RNA	Killed whole viruses
Junin (Argentine hemorrhagic fever)	−RNA	Live attenuated viruses
Japanese encephalitis	+RNA	Killed whole viruses
		Live attenuated viruses[a]
Cytomegalovirus	ds DNA	Live attenuated viruses[a]
Dengue	+RNA	Live attenuated viruses[a]

[a] Vaccines are close to release to the public.
[b] The licensed rotavirus vaccine had been withdrawn from market, pending evaluation of risk of intussusception in infants.
[c] In military recruits.

Epstein–Barr virus. They generate short RNAs binding the protein kinase which plays an important role in interferon induction.[70,73]

RNA-based viruses possess a decidedly lower coding capacity than DNA viruses. They frequently, encode only several proteins which are indispensable for viral genome replication and virion formation. In spite of that, some RNA viruses have also developed specific strategies to evade immune response. They can down-regulate the MHC class I synthesis or interfere with MHC class II expression.[70] However, neither of these mechanisms explains why viruses using RNA to store their genetic information can so easily generate immune escape variants or drug-resistant mutants. Certainly, RNA-based viruses possess some additional strategy that they apply to escape from immune or drug surveillance. Several lines of evidence suggest that unusual genetic variability plays this role.

3. MECHANISMS GENERATING GENETIC VARIABILITY OF RNA-BASED VIRUSES

The first observations indicating the unusual heterogeneity of RNA viruses come from the pioneer studies conducted by Weissmann and co-workers on phage Qbeta. They found that each viable phage genome in a multiply passaged population differs in one or two positions from the average sequence of the parental population.[79,80] This led Weissmann to conclude that the Qbeta 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.[81]

Table II. Examples of Antiviral Drugs

Drug	*Virus*	*Mechanism of action*
Acyclovir	HSV, VZV	DNA synthesis terminator
Gancyclovir	CMV, acyclovir-resistant HSV, and VZV	DNA synthesis terminator
Vidarabine	HSV, VZV	DNA polymerase inhibitor
Idoxuridine; trifluridine	HSV (ocular HSV infections)	Disturb viral DNA replication, polymerase inhibitor
Foscarnet	CMV, HSV, VZV	DNA polymerase inhibitor
Amantadine	Influenza A virus	Ion channel inhibitor
Rimantadine	Influenza A virus	Ion channel inhibitor
Ribavirin	RSV, HCV	Multiple mechanisms (among other increases the rate of viral genome mutation)
Zidovudine	HIV	DNA synthesis terminator
Didanosine	HIV	DNA synthesis terminator
Stavudine	HIV	DNA synthesis terminator
Zalcitabine	HIV	DNA synthesis terminator
Lamivudine	HIV, HBV	DNA synthesis terminator
Abacavir	HIV	DNA synthesis terminator
Nevirapine	HIV	Reverse-transcriptase inhibitor
Efavirenz	HIV	Reverse-transcriptase inhibitor
Delavirdine	HIV	Reverse-transcriptase inhibitor
Ritonavir	HIV	Protease inhibitor
Indinavir	HIV	Protease inhibitor
Nelfinavir	HIV	Protease inhibitor
Saquinavir	HIV	Protease inhibitor
Lopinavir	HIV	Protease inhibitor
Amprenavir	HIV	Protease inhibitor
Interferon alpha	HBV, HCV, HPV	Complex

HSV, herpes simplex virus; VZV, varicella-zoster virus; CMV, cytomegalovirus; RSV, respiratory syncytial virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HBV, hepatitis B virus; HPV, human papillomavirus.

During the next decades it became increasingly clear that an individual RNA virus or retrovirus does not form a homogenous population but circulates in a host organism as a pool of genetically distinct variants.[5–10] To describe such a complex structure of virus population the concept of quasi-species was applied. The term quasi-species was originally introduced by the authors of the RNA world hypothesis to depict replicons that could function in the early stage of the evolution of life.[82,83] The quasi-species was understood as a mixture of self-replicating RNA molecules composed of one or several master sequences and substantial amounts of closely related sequences that replicate almost as fast and almost as faithfully as the master. Accordingly, the virus quasi-species was defined as a whole population of phylogenetically related variants which are present in a single infected organism.[7–10] There are three models of virus quasi-species: biological, chemical, and physical. From the biological standpoint a quasi-species is a phenotypic expression of the population dominated by one or several master sequences. Chemically, the quasi-species is a rated distribution of related non-identical RNA molecules. Physically, the quasi-species is a cloud in sequence space formed by all possible variants of a genomic sequence. For a single stranded RNA virus or retrovirus of 10 kb (e.g., HCV or HIV) the sequence space is $4^{10,000}$.

The cloud representing a quasi-species' distribution usually moves within the sequence space as long as new viral variants are produced and subjected to a continuous process of competition and selection. If positive selection is applied, the process of differentiation of virus population is accelerated and certain mutations may become fixed. On the other hand, negative selection can reduce genetic polymorphism by eliminating some viral variants. Consequently, the level of heterogeneity of virus quasi-species is not simply related to the mutation rate but depends on many different factors shaping the viral population.[84,85]

As a result of a systematic analysis of viruses appearing in the infected organisms a large number of more or less complicated modifications occurring within their RNA genomes were identified. In the most general terms, they can be divided into two basic groups: point mutations and more complex rearrangements of genomic molecules. Modifications of the first type are introduced into the viral genome during its replication or post-replicationly, whereas modifications of the second type are the products of RNA recombination or genetic reassortment. The main mechanisms responsible for their occurrence are presented below.

A. Point Mutations

1. Mechanisms Generating Point Mutations

There are many different processes because of which point mutations appear in the genomic RNA molecules. They can arise after mutagene treatment or be introduced by cellular enzymes involved in posttranscriptional RNA modification. However, under physiological conditions, the number of mutations of this type is incomparably smaller than the number of changes introduced during an imprecise replication of the genomic molecules. RNA-based virus replicases lack proofreading activity ($3'–5'$ exonuclease activity) and there is no doubt that error-prone replication is the main source of point mutations in the RNA genome.

2. Error-Prone Replication

The RNA-based virus' life cycle requires a multiple replication of genetic material prior to the production of progeny virions. This process is especially complicated in the case of retroviruses.[86] Initially, single-stranded genomic RNA (ssRNA) is copied into a single-stranded DNA (ssDNA), which at the next stage serves as a template for the synthesis of a double-stranded DNA (dsDNA). Both processes are catalyzed by retroviral reverse transcriptase (RT), which is both a RNA- and DNA-dependent DNA polymerase. Then dsDNA is integrated with the host genome. After integration, the proviral DNA is copied together with the host's genetic material by the cellular replication complex. At the last stage, the retroviral genomic RNAs are transcribed by the host RNA polymerase. Therefore, a question arises: which of the four mentioned processes (i.e., (i) RNA-dependent DNA synthesis, (ii) DNA-dependent DNA synthesis, (iii) host DNA replication, or (iv) host RNA transcription) is responsible for the error-prone replication of the retroviral genome? With certainty, it is not host genome replication, in the course of which mutation frequency amounts to hardly 10^{-10}–10^{-12} mutations per inserted nucleotide[87] and, therefore, we can omit it in our further considerations. Contrary to host DNA replication complexes, RTs like cellular RNA polymerases (transcriptases) lack proofreading activity, hence the reaction catalyzed by them is the main source of mutations.[5,6,88] From the data collected so far, it follows that RTs copy genomic molecules with a precision similar to that of host transcriptases synthesizing cellular RNAs. Moreover, it has been proved that RTs introduce a similar number of modifications during RNA-dependent and DNA-dependent DNA synthesis. Thus, it may be assumed that all three processes contribute to retrovirus genetic variation in a similar degree. The first two processes (i.e., ssDNA and dsDNA syntheses) are catalyzed by RT, so this enzyme is responsible for the introduction of approximately 70% of point mutations whereas host transcriptases account for the remaining 30% of modifications.[6]

The scheme representing the replication of the RNA virus genome is decidedly simpler. The latter can be composed of positive-sense RNA ((+)RNA—sense RNA encoding viral proteins), negative-sense RNA ((−)RNA—antisense RNA), or double stranded RNA (dsRNA). The entire RNA replication process is catalyzed by RNA-dependent RNA polymerase (RdRp). RdRps are formed by virus encoded proteins (e.g., polymerase, helicase) and some host factors. Depending on the character of the RNA molecule/molecules forming the viral genome, during the first stage of replication RdRp

copies (+)RNA strand into (−)RNA strand (in plus-stranded RNA viruses), (−)RNA strand into (+) strand (in minus-stranded RNA viruses) or double stranded RNA (in dsRNA viruses). In short, the plus strand is used as a template to produce the minus strand and vice versa. During the last step of replication, either (+)RNA, (−)RNA, or dsRNA is amplified and then packed into virions.

It was estimated that the frequency at which RTs or RdRps introduce mutations into a newly synthesized nascent strand ranges from 10^{-2} to 10^{-6} mutations per incorporated nucleotide.[88–91] Theoretically, this means that as many as 100 nucleotides can be incorporated incorrectly during one replication cycle of a genome approximately 10^4 nucleotides in length. However, the accuracy with which viral polymerase copies genomic molecules also depends on RNA primary and secondary structure.[90] After taking into consideration the above factors, the frequency of viral genome mutations *in vivo* was established. It is approximately 10^{-4}–10^{-5} mutation per incorporated nucleotide.[88–91] For some retroviruses it was noted that mutation frequency measured in the *in vitro* systems is usually higher than *in vivo*. This indicates that there may exist hypothetical cellular factors increasing replication precision.[92]

The most frequently occurring mutations are nucleotide substitutions. Among them, transitions (the exchange of pyrimidine into pyrimidine or purine into purine) constitute approximately 80% of mutations, whereas the remaining 20% are transversions (the exchange of the pyrimidine into purine and vice versa). Out of various substitutions A to G is most frequently observed.[6,93,94] It occurs when polymerase introduces U instead of C into the newly synthesized strand. Because U can form a weak, although stable base pair with G, the viral replication complex is not paused and continues nascent strand elongation. The remaining mutations, that is: frameshifts, simple deletions, deletions with insertions, and duplications are definitely more rare.[89–95] For example, the conducted *in vivo* studies revealed the following distribution of point mutations within the HIV genome: substitutions, 81%; frameshifts, 13%; deletions, 4%; deletions with insertion, 2%.[93–95] The putative mechanisms for introduction of some mutation types are shown in Figure 4.

What does, however, the established value of mutation frequency (10^{-4}–10^{-5} per incorporated nucleotide) mean for the typical RNA-based virus, whose genome length is 10^4 nucleotides (e.g., HCV or HIV)? To demonstrate it, some simple mathematical calculations have to be conducted. First of all, it has been calculated that the maintainence of frequently observed virus titers of 10^2–10^4 infectious viruses per milliliter of plasma requires the production of 10^{10}–10^{12} new infectious virions daily. To produce such a high amount of viral particles, virus polymerase has to use as many as 10^{14}–10^{16} nucleotides (10^{10}–$10^{12} \times 10^4$). It is possible since the half-life of individual virions varies from several minutes to several hours, depending on the type of virus. After that time, 50% of virions are degraded and their components can be used again to build new viral particles. To determine how many nucleotides are misincorporated into the viral genome during 1 day we have to multiply the total number of nucleotides used to synthesize RNA genomic molecules by the mutation frequency, thus obtaining 10^9–10^{12} misincorporated nucleotides daily (10^{14}–10^{16} multiplied by 10^{-4}–10^{-5}). Finally, if we divide the number of misincorporated nucleotides by the length of the viral genome we will learn how frequently each individual nucleotide forming genomic RNA can be exchanged to another during 1-day infection, that is 10^9–$10^{12}/10^4 = 10^5$–10^8 times per day. This result well illustrates how many viral variants can be produced in the infected organism and consequently what an enormously high evolutionary potential error-prone replication gives to RNA-based viruses.

Here, it should however be pointed out that mutation frequency is a parameter which only characterizes the accuracy of the polymerase without taking into account the important features of the genome (its size or its rate of replication). Consequently, it cannot be directly used in modeling the genetic structure or evolution of the population. Mutation rate (expressed per genome per genome replication) is a parameter, which much better describes the evolutionary capacity of the given population.[85] It was proposed that every group of organisms displays a characteristic value of mutation rate.[96] It is approximately 0.0034 for DNA-based microorganisms (including viruses or

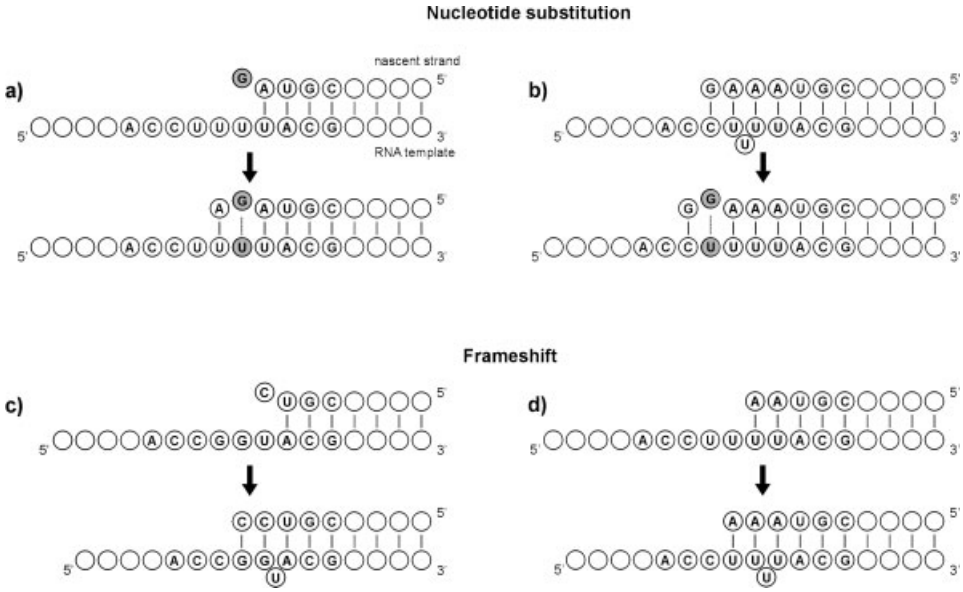

Figure 4. Putative mechanisms used by viral polymerase to introduce point mutations into genomic molecules. In each panel, small circles represent single nucleotides forming the RNA template (lower chain) and nascent strand (upper chain). **a**: Substitution resulting from nucleotide misincorporation. Polymerase added G instead of A. Because G can form a base-pair with U, polymerase is not paused but continues nascent strand elongation. **b**: Substitution resulting from bulge formation. During nascent strand synthesis, a single-nucleotide bulge is formed on the RNA template within a short poly-U stretch. As a result, polymerase does not add one A into the nascent strand. If the perfect double-stranded region is regenerated, the last U present in the poly-U stretch base-pairs with G and polymerase continues nascent strand elongation. **c**: Frameshift resulting from nucleotide misincorporation and consecutive bulge formation. Polymerase introduced C instead of A. It can pause the enzyme and terminate nascent strand synthesis or the misincorporated nucleotide can base-pair with a next nucleotide within the template because of a single-nucleotide bulge formation. **d**: Frameshift resulting from bulge formation. During nascent strand synthesis polymerase reaches the short poly-U tract. If a single-nucleotide bulge is formed on the RNA template polymerase can add only three A (instead of four) and continue nascent strand extension (so-called polymerase slippage).

bacteria) and approximately 0.01 for higher eukaryotes. Recently, Drake and Holland made an interesting attempt to estimate the mutation rate in positive- and negative-strand RNA viruses by applying a simple linear equation that reflects the iterative replication of the single-stranded genome.[85] According to their calculation, it varies between 0.13 and 1.15. Elena and Moya found a similar value of mutation rate, 1.2, by analyzing the data obtained for vesicular stomatitis virus only.[97]

The mutation rate established for RNA viruses is much higher than for DNA-based organisms. Holland and co-workers suggested that its 2.5-fold increase (e.g., by chemical mutagenesis) is sufficient to extinguish a virus population.[98] This observation has important medical consequences. It means that one can successfully fight virus infections by using compounds which can specifically increase the mutation rate of the virus' genomes.

3. Post-Replicational Modifications

The newly synthesized genomic RNA can be further mutagenized in the host cells, although this process is decidedly less effective than error-prone replication. Some changes within viral genomes are introduced by physical or chemical mutagenesis, for example: by UV irradiation, ionizing irradiation, treatment with compounds modifying purine and pyrimidine bases, or by application of nucleoside analogues as antiviral drugs.

Before encapsidation viral genomic molecules can also serve as substrates for enzymes modifying cellular RNA, for example, for those involved in RNA editing.[99] The latter process was

observed in mRNAs, tRNAs, and rRNAs, in mitochondrial, chloroplast, and nuclear RNAs as well as in viral RNAs.[99] Usually, RNA editing is put into two categories based on its mechanism. The first one, called insertion/deletion editing, changes the length of the target RNA. The second category of RNA editing leads to nucleotide substitutions. It most frequently consists of adenosine or cytosine deamination by specific cell enzymes (deaminases, e.g., an enzyme called ADAR—adenosine deaminase that acts on RNA).[100,101] It was found that some viral RNAs are suitable substrates for ADAR which catalyzes the adenosine deamination to inosine. Inosine, like guanosine, prefers to pair with cytidine; therefore, deamination events result in A to G substitutions. The enzyme was identified in a wild spectrum of organisms from metazoan to mammals. It was demonstrated that in some cases even up to 50% of A present in viral RNAs can be modified by ADAR. The function of highly modified viral RNAs remains unclear.[102,103] At present, the best studied example of editing in RNA viruses is the modification of the amber stop codon (UAG) to a tryptophan codon (UGG) in hepatitis delta virus (HDV).[104] In this way the virus possessing a single open reading frame in its genome is able to produce two proteins. The shorter one is essential for HDV replication whereas the longer one is for its packaging.[105]

B. More Complex Rearrangements of the Viral Genome

There are two general mechanisms by which some more profound changes are introduced into the viral genome: RNA recombination and genetic reassortment. The first phenomenon corresponds to recombination occurring in DNA-based organisms, whereas the second can be compared to the exchange of parental chromosomes during meiosis.

1. Genetic RNA Recombination

RNA recombination was first discovered in poliovirus.[106,107] Then its biological role and molecular bases were extensively studied in other picornaviruses,[108] (+)RNA viruses,[109,110] and retroviruses.[111,112] As a result, the opinion arose that this mechanism of genetic material exchange operates only in viruses whose genomes are composed of positive-sense RNA. Such a view was slightly modified when several articles describing non-homologous recombination events in influenza virus ((−)RNA virus)[113] or in Φ6 phage (dsRNAvirus)[114,115] were published. Even though these reports clearly demonstrated the potential of both types of viruses to recombine, they did not change the general opinion that homologous recombination, very frequent in (+)RNA viruses and retroviruses, does not occur in (−)RNA and dsRNA viruses. A significant turning-point in our thinking about RNA recombination came about in 1998 when Suzuki and co-workers showed intragenic homologous crossovers in rotaviruses—dsRNA viruses possessing a segmented genome.[116] However, the final evidence that RNA recombination is a mechanism universally used by all RNA viruses was provided as late as in 2002 by Plyusnin and colleagues.[117] While studying Tula hantivirus (a representative of (−)RNA viruses) they identified a naturally generated putative homologous recombinant. Based on this observation they created an experimental system in which the identical Tula hantivirus was reconstructed by homologous recombination.

At present, it seems that each RNA-based virus is capable of recombining, although the evolutionary implications of this fact for different types of RNA viruses remain to be determined. RNA recombination was observed for human, animal, plant, and bacterial viruses.[109] The exchange of genetic material most frequently took place within a viral population, although it also occurred between different viral strains or between different viruses. Additionally, it was shown that viral RNA can recombine with host RNA[113,118] as well as with transgenic mRNA that is formed in cells expressing viral genes.[119] All these data revealed that RNA recombination is one of the major factors responsible for the emergence of new, often dangerous viral strains or species.

Despite extensive studies, the molecular mechanism of RNA recombination is still not well understood. Initially, two completely different models of genetic RNA recombination were proposed.

The first one assumed that viral recombinants arise as a result of breakage and rejoining of nucleic acid molecules. The same mechanism was proved for DNA recombination. The second one; the so-called copy-choice mechanism assumed that recombination takes place during viral genome replication, when the polymerase engaged in this process switches from one RNA template to another[111,120–122] (Fig. 5). Nowadays, it is generally accepted that RNA recombination occurs in compliance with the assumptions of the copy-choice hypothesis.[123–130] There are, however, reports indicating that an alternative mechanism of recombination may also operate in certain specific situations.[131]

Based on the structure and function of RNA molecules, two types of genetic RNA recombination were distinguished: homologous and non-homologous[109] (Fig. 6). Homologous RNA recombination involves two identical or similar molecules (or two molecules which, although different, do possess a sufficiently long region of homology) and is called precise if recombinant junction sites are located accurately at the corresponding nucleotides, or imprecise when junction sites occupy different positions within the recombining molecules.[124,130] As a result of precise crossovers parental molecules are regenerated, whereas imprecise recombination produces molecules in which some sequences are duplicated or deleted. Homologous recombination may lead both to viral genome stabilization and destabilization. On the one hand, homologous recombination can repair a truncated or incorrectly synthesized gene fragment (Fig. 7A), but on the other hand, it may deepen the process of viral genome diversification (Fig. 7B).

Non-homologous recombination occurs between two various RNA molecules and generates products that differ distinctly from parental molecules.[125,126,128,130] As a result, non-homologous recombinants are frequently dysfunctional and they rarely accumulate *in vivo*. Sometimes, however, non-homologous recombination can produce a new viral strain or species, possessing some advantageous features enabling it to compete successfully with other pathogens. Non-homologous recombination may, therefore, play an especially important role in virus evolution. The appearance of thousands of variants enables the selection and replication of the most adaptable ones, because of which the virus can survive under unfavorable conditions.

2. Recombination in Retroviruses

Retroviruses are spherical enveloped viruses approximately 100 nm in diameter. The retroviral genome is composed of two identical or nearly identical (+)RNA molecules approximately 7–10 kb in length. It may, therefore, be said that retroviruses are ''pseudodiploid'' microorganisms.[86]

The studies on retrovirus genome replication revealed that, occurring according to the copy-choice mechanism, recombination plays a very important role in the virus life cycle.[6] Not only does it

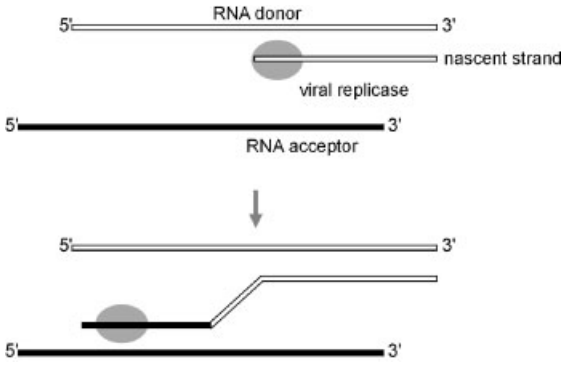

Figure 5. Copy-choice hypothesis. Copy-choice hypothesis assumes that viral recombinants are generated during the replication process. Viral polymerase (represented by a gray oval) starts nascent strand synthesis on one template called RNA donor and then it switches to another template called RNA acceptor. In consequence, the resultant recombinant sequence derives from two different RNA templates.

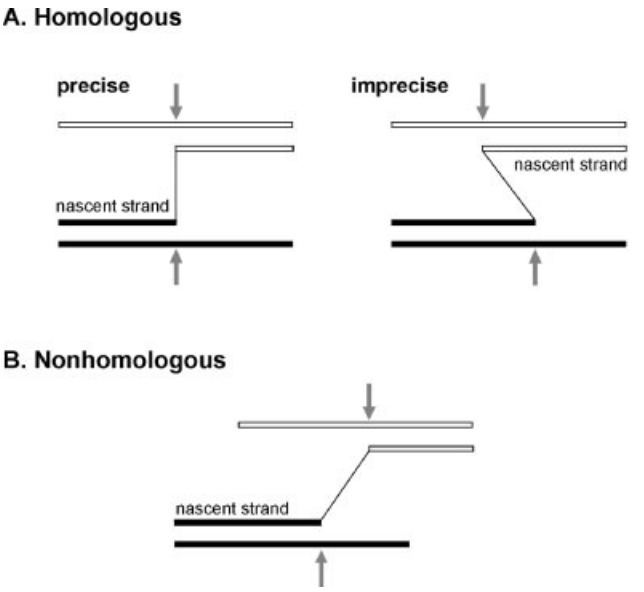

Figure 6. Two basic types of RNA recombination. Depending on RNA structure and function two basic types of RNA recombination were distinguished: homologous (**A**) and non-homologous (**B**). Substrates for homologous recombination are two identical or similar RNA molecules. Homologous recombination is called precise if recombinant junction sites (indicated by arrows) are located accurately at the corresponding nucleotides or imprecise when junction sites occupy different positions within the recombining molecules. Precise homologous recombinants are difficult to observe since they are identical with parental molecules. Imprecise recombination products are easier for identification as some portion of their sequence is duplicated or deleted (in comparison with parental molecules). RNA recombination is called non-homologous if RNA donor and acceptor are different. Non-homologous recombinants differ significantly from parental molecules and, being dysfunctional they rarely accumulate *in vivo*.

contribute to the generation of the extremely high genetic variability of retroviruses but it is also required for the synthesis of dsDNA, which is integrated with the host genome.[86] As it is shown in Figure 8 retrovirus RTs have to make at least two template switchings to copy genomic ssRNA into proviral dsDNA. Accordingly, it was proposed that retroviral RTs are evolutionarily selected to mediate recombination.[132]

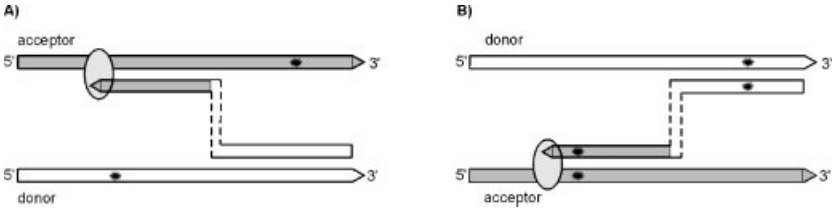

Figure 7. Non-homologous recombination can stabilize as well as destabilize the viral genome. The above scheme assumes the existence of two genomic molecules, which differ from the wild type genome by a single nucleotide. The first molecule possesses a single mutation placed close to its 3′-end, whereas the second molecule has a single mutation located close to the 5′-end (the mutations are marked with stars). Both molecules can recombine and their recombinant junction sites are located in their central portion. **A**: If polymerase (represented by a gray oval) starts nascent strand synthesis on the template lacking a mutation at the 3′-end (RNA donor—in white) and then switches to the acceptor template (in gray) with no mutation at the 5′-end, a wild type genome is regenerated. **B**: If the polymerase begins nascent strand synthesis on the molecule carrying a mutation at the 3′-end (RNA donor) and afterwards it switches to the template mutated within the 5′-end, a recombinant possessing simultaneously two mutations will be generated.

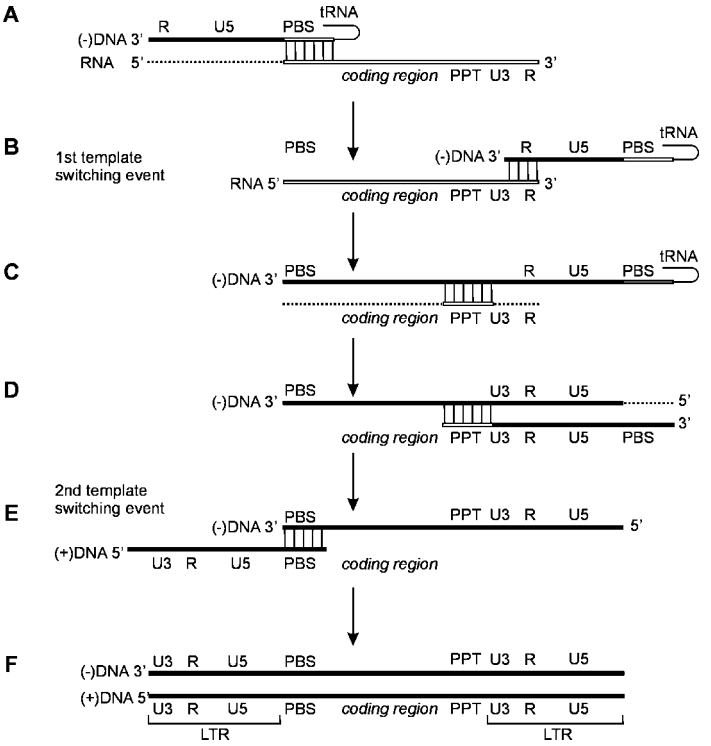

Figure 8. HIV genome replication. During HIV genome replication, genomic ssRNA is converted into dsDNA (white and black boxes represent RNA and DNA molecules respectively). **A**: Three different regions can be distinguished within HIV genomic molecule: the 5′-non-coding region [composed of the primer binding site (PBS), sequences U5 and R), the central coding fragment (not in scale), and the 3′-non-coding region [composed of the polypurine tract (PPT) and sequences U3 and R]. To initiate replication HIV RT uses tRNA as a primer. tRNA binds to PBS and HIV RT extends it, at the same time degrading the 5′-non-coding region of the genomic RNA (degraded fragments of genomic RNA are represented by a doted line). **B**: When HIV RT reaches the 5′-end of the genomic RNA the first template switching event occurs. The HIV RT–(−)DNA strand complex is transferred from sequence R (located at the 5′-end of the genomic RNA) to an identical sequence R located at the 3′-end in the same or another genomic RNA (two identical or similar genomic RNAs are present within the HIV nucleocapsid). **C**: HIV RT continues (−)DNA synthesis and simultaneously it degrades the remaining portion of the genomic RNA, except the PPT sequence. **D**: During the next stage HIV RT initiates (+)DNA synthesis using PPT as a primer. When HIV RT reaches the 5′-end of the (−)DNA strand it degrades a fragment of the tRNA primer and pauses. **E**: At this moment the second template switching event occurs. The HIV RT–(+)DNA complex is transferred to the PPT sequence located in the (−)DNA strand. **F**: HIV RT extends both (+)DNA and (−)DNA strands. Resultant dsDNA is integrated with the host genome. It is composed of the coding region flanked by PBS and PPT as well as by two identical sequences called long terminal repeats (LTR).

Since retroviruses are pseudodiploid, both intra- and intermolecular recombination crossovers are observed during their genome replication. It was demonstrated that RTs introduce shorter or longer insertion, duplication, and deletion into a viral genome very frequently.[6,111,112,123,133] For example, RT can very efficiently remove direct sequence repeats introduced into genomic RNA. Depending on the length of the repeated sequence, recombination frequency varies from 40 (for ca. 100 nucleotide sequence) to 90% (for ca. 700 nucleotide sequence).[133]

Homologous as well as non-homologous template switching events are usually supported by short stretches of homology, but sometimes there is no homology within the crossover region. As a result of the very high rate of RT mediated recombination, genetic material of progeny viruses is derived from both genomic RNAs. In addition, RT mediated non-homologous recombination is most likely used to capture oncogenes into the retroviral genome.[134] Because sequence similarity between recombining molecules strongly enhances template switching by RT, short regions of homology are usually observed at the junctions of retroviral genome and oncogene.

3. Recombination in RNA Viruses

Investigations conducted with picornaviruses and coronaviruses are of special historical importance. They created proper grounds for further RNA recombination studies, causing recombination to become a fact in the RNA world. Picornaviruses are small, icosahedral, (+)RNA viruses infecting humans and animals.[135] In the early 1960s, it was shown for the first time that mixed infections with two strains of poliovirus, each carrying a specific genetic marker, resulted in progeny exhibiting simultaneously both features characteristic to parental viruses.[106,107] A similar phenomenon was observed for foot-and-mouth disease virus (FMDV).[108] During a single replication cycle approximately 10–20% of viral genomes underwent homologous recombination.[136] Several years later Kirkegaard and Baltimore demonstrated that the suppression of poliovirus genome replication inhibits RNA recombination.[120] This observation provided the first experimental evidence supporting the copy-choice mechanism of RNA recombination. The proposed model of genetic recombination in picornaviruses assumes that template switching events occur preferentially but not exclusively during (−) strand synthesis. Recombination crossovers are roughly randomly distributed along genomic RNA, whereas recombination frequency strongly depends on the extent of similarity between parental RNAs.[137,138].

Coronaviruses are single-stranded, (+)RNA viruses with an extremely large non-segmented genome (from 27 to 31 kb) comprising 7–10 genes.[139] Genetic RNA recombination in coronaviruses was especially intensively investigated by using mouse hepatitis virus (MHV). Studies involving MHV disclosed a very interesting but, at the same time, complicated picture of genetic RNA recombination. In coronaviruses, each gene is expressed from a separate mRNA. Interestingly, mRNA molecules are synthesized in discontinuous transcription resembling the RNA recombination process. First, RNA polymerase synthesizes a 70–90 nucleotide leader sequence (derived from the 5′-end of genomic RNA). Then the polymerase–leader complex leaves the template and restarts RNA synthesis on one of the intergenic transcription promoters. Resultant mRNAs have a leader sequence at the 5′-end and are 3′-coterminal. Initially, it was proposed that discontinuous transcription occurs during positive-strand synthesis, as genome-length negative strands were only found in coronavirus infected cells.[109] After several years, Sawicki and co-workers verified this model by showing that transcriptionally active negative-strand RNAs corresponding to each subgenomic mRNA are also formed during infection.[140] Consequently, they proposed that discontinuous transcription occurs during negative strand synthesis.

The mechanism of coronavirus mRNA formation by discontinuous transcription suggests that at least some viral polymerases are, like retroviral RTs, naturally selected to mediate template switching events. Indeed, studies involving MHV demonstrated that approximately 25% of genomic molecules are recombinants.[141,142] Especially frequent recombination crossovers are observed in the MHV hypervariable region (within the envelope protein encoding sequence).[143]

Although the discussion of recombination in plant viruses is outside the scope of this review the great impact of plant virus studies on our understanding of RNA recombination should also be noted. Especially interesting data were obtained with brome mosaic virus (BMV), a model positive-sense RNA virus of plants.[144] Unlike any other, the BMV-based recombination system enables homologous[145–147] and non-homologous[128,148] recombination studies at both protein[121,122] and RNA levels.[128,145–149] In consequence, BMV is the virus for which the structural requirements for genetic RNA recombination are most precisely established.[124,125] Interestingly, it was observed among others that the same RNA structural elements which support *in vivo* non-homologous recombination in BMV are able to induce *in vitro* template switching by HIV RT.[150]

There are several other RNA viruses in which genetic recombination was investigated and some interesting preliminary observations were made. Mindich and co-workers found that in bacteriophage Φ6 (double-stranded RNA virus with a three-segmented genome) recombination may occur inside procapsids, where viral polymerase synthesizes dsRNA using a (+)RNA strand as a

template.[114,115] Recombination events involve different RNA segments since they were classified as non-homologous, although recombinant junction sites are usually located within short regions of homology between recombining molecules.[114,115] Similar observations were made during nodavirus studies.[151] However, in the latter viruses, two other factors, in addition to local homology, seem to influence template switching by viral polymerase: template secondary structure, which may bring recombinant junction sites close together, and promotor-like sequences, which can directly bind viral polymerase. Different results were obtained by Raju and co-workers who found that in Sindbis virus homologous sequences are not required for recombination to occur.[152] Recombinant junction sites are randomly distributed along donor and acceptor templates, just like it was found in picornaviruses.

In general, the majority of data indicate that RNA viruses exchange their genetic material as predicted in the copy-choice hypothesis.[109,112,123–125,130] The location of recombinant junction sites and recombination frequency depend on the specific properties of viral replicases and on the primary- and secondary-structure of the recombining molecules.

4. Copy-Choice Mechanism of Genetic RNA Recombination

Though the copy-choice hypothesis does not explain the molecular bases of recombination process, it does, however, permit to distinguish its three main stages (Fig. 9). During the first stage, nascent strand synthesis on the template called RNA donor is paused. There are many factors which can induce the stopping of viral replicase on the RNA donor, for example: the lack of template continuity, the presence of specific sequences or structural motifs, a nucleotide misincorporation or genomic RNA interactions with a protein not participating in replication (for example, capsid protein). At the second stage, the replicase and nascent strand are released from the RNA donor (as a complex or separately) or the replicase moves backward on the template releasing the 3′ end of the newly synthesized strand. During the third stage, replicase and nascent strand are transferred to a new template called the acceptor (ssRNA in RNA viruses and retroviruses or ssDNA in retroviruses). The scenario of that process depends on the course of the second stage. If replicase as well as the nascent strand are released from the RNA donor as a complex, they can be entirely transferred onto the acceptor template. The factors enabling this process are nascent strand hybridization to a complementary sequence present in the acceptor RNA/DNA, or replicase binding to the promoter or promoter-like sequence on the acceptor RNA/DNA. If, during the second stage, replicase and nascent strand leave the donor template separately, the replication complex has to be reconstituted on the acceptor template. In such a situation the 3′-end of the nascent strand can hybridize to a complementary sequence located within the acceptor template and then replicase can use it as a primer to reinitiate RNA/DNA synthesis. If, during the second stage, replicase moves backward on the RNA template, the RNA donor can be degraded (for instance, by ribonuclease H constituting part of RT). In consequence, the 3′ end of the nascent strand is released. It can hybridize to a complementary sequence present in the RNA/DNA acceptor, enabling replicase jumping to a new template.

5. Genetic Reassortment

In viruses with segmented genomes there is an additional mechanism generating genetic diversity, termed reassortment.[153,154] Genetic reassortants can form when at least two different viruses with multipartite genomes infect the same cell and exchange their genetic material. Reassortment is most likely to occur at the stage of morphogenesis at which segments are selected from intracellular pool for packaging. Because of the exchange of the single genome segment virus can gain totally new potentials, frequently a dramatic antigenic shift is observed.[155,156] Genetic reassortment between many ss(−)RNA viruses (e.g., members of Orthomyxoviridae,[157–159] Bunyaviridae[160–162], and Arenaviridae[163] families) and between dsRNA viruses (e.g., representatives of Reoviridae[164,165] family) was demonstrated. Over the years, it has been most extensively studied in orthomyxoviruses.

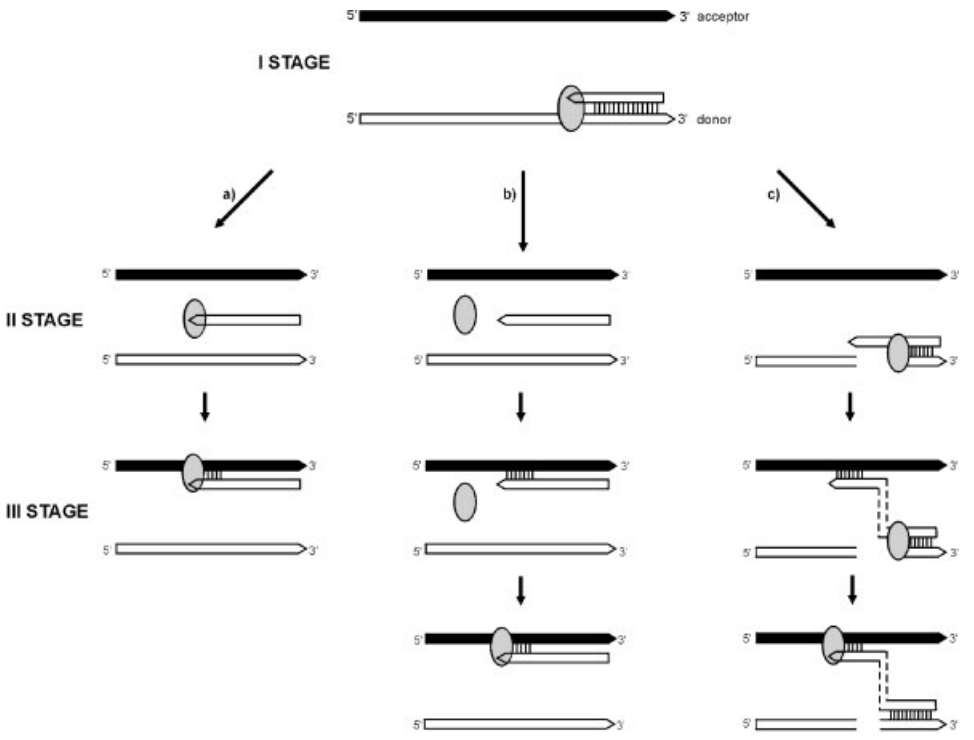

Figure 9. Copy-choice mechanism of RNA recombination. The data accumulating during the last years suggest that recombination events can occur according to at least three different scenarios. In each of them three basic stages can be distinguished. During the first stage, common to all three scenarios, viral replicase (represented by a gray oval) is paused on the donor template (in white). Scenario (**a**) assumes that in the second stage the replicase and the nascent strand are released from the RNA donor as a complex. During the third stage the replicase–nascent strand complex binds to the RNA acceptor and using it as a template continues RNA synthesis. Scenario (**b**) presumes that, in the second stage, replicase and nascent strand leave the donor template separately. In the third stage, the 3′-end of the nascent strand hybridizes to the acceptor template and replicase uses it as a starter to resume RNA synthesis. Scenario (**c**) postulates that in the second stage viral replicase moves backward on the donor template releasing the 3′-end of the newly synthesized strand and at the same time degrading a portion of the RNA donor. During the third stage, the 3′-end of the nascent strand hybridizes to the complementary region present in RNA acceptor. Finally, viral replicase moves forward and switches to the acceptor template.

The Orthomyxoviridae family comprises enveloped viruses with a segmented genome composed of single-stranded, negative-sense RNA.[166] The family contains two genera: influenza A and B viruses and influenza C virus. Genomes of influenza A and B are composed of eight segments, whereas the genome of influenza C contains seven segments. The influenza A, B, and C can be distinguished on the basis of antigenic differences between nucleocapsid and matrix proteins. Influenza A viruses are further divided into subtypes based on the antigenic nature of their hemagglutinin and neuraminidase glycoproteins. In addition to humans, influenza A viruses naturally infect several other mammals (including swine and horses) and a variety of avian species. It seems that humans are the only natural host for influenza B and C, although there are reports suggesting that influenza C can also infect swine.[166,167]

Genetic reassortment was first observed in 1949 for influenza A viruses during mixed infection of the mouse brain.[168] Further studies demonstrated that all three types of influenza virus (type A, B, and C) can easily produce reassortants *in vivo* (in a laboratory).[169] The exchange of genome segments occurred between homotypic viruses but never between different types.[169,170] More detailed investigations revealed that the frequency of reassortment at any given locus is as high as for independently segregating genes, i.e., 50%.[157–159] This raised an important question whether genetic reassortment is

a random process and how individual segments are packaged to ensure virus infectivity and its ability to replicate. If genomic RNAs are selected for packaging randomly it would mean that out of 416 particles of influenza A virus only one contains a full complement of eight different genes.[171] Donald and Isaacs demonstrated that this is not the case, since in both influenza A and B, 1 in 10 particles is infectious.[172] Consequently it was proposed that influenza virus either contains more than eight segments in the virion or there is some mechanism ensuring the precise selection of the genomic molecules.[173]

Studies involving influenza A, B, and C demonstrated that in nature the frequency of reassortment also depends on virus host range.[156,174–176] This explains why, contrary to influenza B and C, natural reassortants are frequently isolated for influenza A infecting not only humans but also animal and avian species. It was shown that reassortants of avian influenza A viruses with human influenza A viruses were responsible for at least two major pandemics in the XX century. Several observations suggest that swine may serve as ''mixing vessels'' for the generation of human–avian influenza A reassortants.[177] However, there are also data indicating that avian influenza A virus that in 1997, killed several persons in Hong Kong was transmitted directly from poultry to humans.[178] On the other hand, the lack of the animal reservoir for influenza B and C leads to the conclusion that genetic reassortment is not an important factor in the evolution of these viruses. The latter opinion seems to be inconsistent with the recent observation of McCullers and co-workers who identified some natural reassortants of influenza B virus.[175] They have also suggested that reassortment between circulating strains of influenza B may play a role in virus evolution. In general, there are many reports indicating that genome reassortment is one of the major factors affecting genetic plasticity not only in orthomyxoviruses but also in other ss(−)RNA viruses as bunyaviruses and arenaviruses.

The bunyaviruses family comprises over 300 individual virus species possessing trisegmented genomes.[179] Most bunyaviruses are insignificant human pathogens, only a few of them are important causes of human diseases, for example, viruses included in the California serogroup, Riff Valley fever virus, Hantaan virus, or Crimean-Congo hemorrhagic fever virus. Genetic reassortment was demonstrated for many members of the California serogroup.[180–182] Exchange of genetic material occurred only between viruses from the same serogroup. The analysis of bunyavirus isolates suggests that reassortment occurs also in nature.[183]

The Arenaviridae family comprises ss(−)RNA viruses with dipartite genome.[184] Arenaviruses are recognized as clinically important human pathogens. The best studied member of the family is lymphocytic choriomeningitis virus (LCMV). Mixed infection involving several different strains of LCMV showed that all possible combinations of two genome segments generate infectious virus.[185]

dsRNA viruses with segmented genomes form another large group of pathogens using genetic reassortment as an important strategy enabling their rapid evolution. Among them reoviruses are of special medical importance.[186] The Reoviridae family contains nine genera out of which four infect humans: reovirus, orbivirus, coltivirus, and rotavirus. Reo- and orbivirus genomes are composed of ten segments, rotaviruses have 11 segments, whereas coltiviruses have 12 segments. High frequency of genetic reassortment was observed for all members of Reoviridae family.[187–195] Reassortants were readily generated in natural hosts and in tissue culture systems. Most of the collected data indicate that the same general roles that have been found for reassortment between ss(−)RNA viruses apply also for dsRNA viruses reassortment.[196–198] It was shown that genetic reassortment of reoviruses is not a random process.[199] It occurs only within serogroups, and the mechanism by which different segments are selected from cytoplasm remains unclear.

4. CONCLUSIONS

DNA and RNA are the only molecules used by living organisms to encode their genetic information. It is stored in chemically stable DNA whereas decidedly less stable RNA is utilized for its

expression. Such a strategy ensures the genetic stability of DNA-based organisms, and allows them to reach a high level of specialization. RNA viruses and retroviruses are the only known living species using RNA as genetic material. At first glance, it would be expected that they have no chance in the competition with DNA-based organisms possessing stable genomes. However, it is not true. RNA-based viruses have employed the supposed weakness as a very effective weapon in their struggle to survive.[5–10] It is possible because the mechanisms of genetic material replication in DNA- and RNA-based organisms are different. According to the central dogma of molecular biology, the flow of genetic information proceeds from DNA through RNA to proteins. In the case of viruses, this principle concerns only those, whose genome is composed of DNA. In viruses using RNA as the only or main carrier of genetic information, the above scheme is subjected to simplification: RNA → proteins (in RNA viruses), or to extension: RNA → DNA → RNA → proteins (in retroviruses). As a result, RNA-based viruses cannot use the very efficient host replication machinery to copy their genomes. Instead, they encode decidedly less accurate RNA-dependent RNA polymerases (RNA viruses) or RNA-dependent DNA polymerases (RTs-retroviruses). In addition, the mechanisms by which RNA- and DNA-based organisms exchange their genetic material are completely different. In consequence, the mutation rate of the RNA virus genome is several orders of magnitude greater than that observed for the host DNA genome.

The most important factors producing the extremely high evolutionary potential of RNA-based viruses are: (i) the high level of replication—during 1 day 10^{10}–10^{12} infectious virus particles are produced in an infected organism; (ii) error-prone replication—each nucleotide forming the viral genome can be exchanged to another 10^5–10^8 times during a 1-day infection; (iii) homologous recombination—20–80% of viral genomic RNAs are homologous recombinants; (iv) non-homologous recombination—because of non-homologous recombination foreign sequences (derived from other viruses or host cells) can be introduced into a viral genome; (v) genetic reassortment—viruses possessing segmented genomes can exchange individual genes. The part, which each factor plays in generating genetic variability not only differs from virus to virus but is also affected by the host organism. This, however, does not change the RNA-based virus ease of adaptability.

Taking into account the above facts one can say that RNA-based virus infection resembles a complex RNA selection experiment (a kind of SELEX experiment but conducted *in vivo*). During the first stage of infection (acute phase) a large pool of progeny viruses, possessing very diversified genomic molecules is generated. Then viral variants are subjected to selection by the host organism. As a result, the infectious, well replicating, non-neutralizable by the host immune system and drug-resistant mutant can be selected (Fig. 10). Its emergence gives a virus the chance to establish persistent infection.

On the other hand, a pathogenic virus also produces strong evolutionary pressure shaping its host population.[200,201] Selective forces eliminate organisms most susceptible to the virus, giving advantage to those individuals who can restrict infection or eliminate the pathogenic agent. The evolutionary capacity of DNA-based organisms is much lower than that of RNA-based viruses; therefore, the former cannot quickly generate virus-resistant variants. However, it seems that the longer the host population is exposed to a virus, the less sensitive to infection it becomes. That may explain why newly emerging viruses are especially malignant whereas those which have accompanied the human being for a very long time are usually milder. Accordingly, at present infections induced by HIV and HCV are the greatest challenges facing modern virology and medicine. The recently estimated and still growing numbers of people infected with HIV and HCV worldwide are 37 and 170 million,[202,203] respectively.

There are several facts indicating that *Homo sapiens* became a host for HIV not very long ago. HIV probably evolved from a Simian immunodeficiency virus (SIV) infecting anthropoid apes.[204–207] RNA recombination was recognized as a putative factor enabling HIV ancestor to overcome an interspecies barrier at the beginning of the XXth century. The genetic plasticity of retroviruses as well as the lack of adaptation to a host organism, which exerts an enormous selective

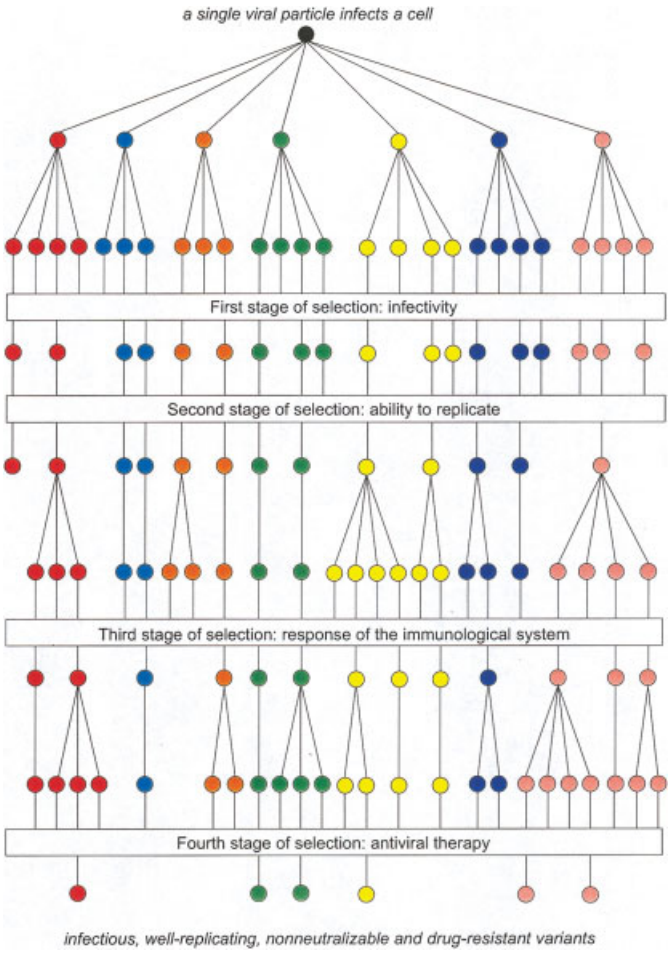

Figure 10. RNA-based virus infection as a complex *in vivo* RNA selection experiment. RNA-based virus infections can be compared to RNA selection experiments that are routinely done *in vitro* in many laboratories around the world. During the first stage, a single viral particle (wild-type virus—represented by a black dot) infects the cell and starts to replicate. As a result, a large pool of very diversified viral particles (represented by color dots) is generated. Practically each virus differs from the other as well as from the wild-type form. When viral particles leave the cell they are subjected to selection by the host organism. First of all, they must be infectious to enter a new cell—the first stage of selection. Those viruses that have been able to infect new cells have to replicate their genomes and produce progeny; in the other case they are eliminated—the second stage of selection. After a few days, replicating viruses are subjected to selection by the host immune system. As a result, immune escape mutants are generated—the third stage of selection. Finally, the infected patient is subjected to antiviral therapy and only drug-resistant mutants can replicate and spread further—the fourth stage of selection. This way, the four-step selection generates infectious, replicating, non-neutralizable, and drug-resistant viral variants.

pressure on the virus, are presumably major determinants of the great evolutionary potential and the high pathogenicity of HIV.

Several lines of evidence suggest that genetic variability and lack of equilibrium with the host are also the primary factors involved in HCV persistence and consecutive chronic hepatitis C development (one of the major causative agents of cirrhosis and hepatocarcinoma).[208–210] HCV was identified as late as in[211] 1989 and classified as a member of the Flaviviridae family. Interestingly, HCV also shares properties with picornaviruses and plant potyviruses. This has led to speculation that HCV might represent an evolutionary link between animal and plant viruses.[212,213] The clinical course of HCV infection is rather unusual. It begins with an acute phase, which is asymptomatic in 70% of patients. However, acute hepatitis C is resolved only in 15–30% of infected persons. In the

remaining 70–85% of individuals, acute hepatitis C evolves into chronic infection. Finally, it has been estimated that cirrhosis occurs in 20–40% of chronic hepatitis patients and hepatocarcinoma in approximately 10% of patients.[214]

The presented data suggest that the basic condition of finding efficacious methods of fighting RNA-based viruses is a better understanding of the mechanisms responsible for the extremely rapid evolution of RNA genomes. It is becoming increasingly apparent that the enormous variability of RNA viruses is at the basis of our problems with combating viral infections.

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