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Research initiatives in studies of antiviral resistance and consensus points and recommendations

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1. Introduction

While much knowledge of antiviral resistance has accumulated over the past decade, many areas exist in which further research is warranted. Diseases for which new antiviral therapies are needed include the viral hepatitides, papillomavirus infections, diseases caused by acute respiratory viruses, viral gastroenteritides, and viral myocarditis. The application of molecular biology to the development of novel antiviral agents promises to provide the clinician with the ability to treat diseases for which therapeutic interventions do not currently exist. In addition, development of new methods by which these antiviral agents can be delivered, such as mucosal delivery systems and intramuscular administrations which require only infrequent dosing, will further improve patients' quality of life by minimizing disruption of their daily routines. Moreover, development of these new vectors will allow state-of-the-art medical advances to reach persons in very remote settings throughout the world.

Development of antiviral resistance will surely increase in frequency and significance as these agents are used more widely, creating a need for redundancy among antiviral drugs. This emphasis on new drug development will persist despite the advances in vaccine development. Combinations of antiviral agents will likely be

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used increasingly in antiviral strategies as new drugs are formulated, especially for viruses with persistent and high levels of replication [e.g. human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV)]. Given the limited number of antiviral compounds currently available, the addition of new agents to the antiviral armamentarium should remain the focus of research efforts, rather than the development of new antiviral drugs designed specifically to impair the emergence of resistance. As new antivirals are introduced into the clinical arena, it is important that mechanisms be established that allow the prospective monitoring of emergence of antiviral resistance both at the community level and at specific sites within individual patients.

2. The Herpesviruses

2.1. Herpes simplex virus (HSV)

Of all of the viruses in which antiviral resistance has been studied, HSV is perhaps the best understood. Areas still exist, however, in which additional knowledge, improved surveillance, and alternative strategies are needed. At the molecular level, further genetic characterization of isolates which appear to be resistant to acyclovir (ACV) therapy is needed. By correlating genetic changes with effects on viral thymidine kinase (TK) production or function, this will provide an enhanced appreciation of expression of TK in the clinical setting. Much work also remains in the genetic and biochemical characterization of those isolates in which resistance is mediated by mutations within the DNA polymerase gene. As advances are made in these areas, specific mutations will be more thoroughly correlated with clinical outcome. Taken together, these advances will allow for the development of more precise and relevant biochemical and genetic characterization of HSV isolates.

Rapid assays are needed to complement the plaque reduction assay (PRA), which is the gold standard for the assessment of antiviral resistance in HSV. Ideally, such an assay system would be easy to perform and provide results that are clinically relevant. In addition, these assays would be well standardized between laboratories, with dose response curves being generated by the assay. Such an assay would be performed directly on clinical specimens, eliminating the requirement for re-growth or pre-assay titering of the clinical isolate. In addition, TK enzyme assays with improved sensitivity and more quantitative results are needed to more precisely define phenotypes among those isolates with in vitro resistance to ACV. As these next generations of assays are developed, more laboratories around the world should be encouraged to assess HSV isolates for antiviral resistance.

Good animal models exist for the investigation of HSV infection. The greatest potential for advancement in this area relates to the analysis of more thoroughly characterized mutant viruses in these animal systems. This will provide knowledge

of the in vivo behavior of these isolates. Such information will also be obtained from continued surveillance and more precise phenotypic characterization of clinical isolates from human subjects. As the prevalence of ACV-resistant mutants increases among immunocompromised individuals, clinical surveillance will determine whether disease caused by resistant viruses is occurring in immunocompetent patients.

2.2. Cytomegalovirus (CMV)

The viral UL97 phosphotransferase has been identified as the enzyme which catalyzes the initial phosphorylation of ganciclovir (GCV). However, the intrinsic function of the UL97 gene product and its role in viral replication and pathogenesis is unknown. Elucidation of the function of the UL97 phosphotransferase will provide valuable information regarding the biology of CMV. In addition, mutations in the UL97 gene which confer GCV resistance can then be better evaluated with respect to adverse effects on viral pathogenesis. Further genotypic analyses need to be performed on those CMV isolates with cross-resistance phenotypes, as well as those isolates with mutations in the DNA polymerase. Ultimately, additional antiviral agents that target CMV gene products other than the DNA polymerase need to be developed; examples of potential targets include the viral protease and the viral terminase.

Of all the viruses for which genotypic assays are feasible, CMV holds the most promise for clinical use. This is due to the fact that 85% of GCV-resistant mutants have deletions or point mutations at one of three amino acid sites. Such a polymerase chain reaction (PCR)-based genotypic assay would be very rapid and could be performed directly on clinical specimens. In addition, the enhanced sensitivity of such an assay could help to determine if UL97 mutants preexist in the absence of GCV therapy, as is the case with HSV. This in turn would have implications in the development of new strategies to prevent the emergence of CMV resistance.

While good animal models are available for the study of HSV infection, those that exist for study of CMV are largely inadequate. A SCID-hu mouse model is available but is very labor-intensive. In addition, the animal must be killed for any endpoint measurement, and the applicability to human disease has been questioned. An ocular SCID mouse model has also been developed and can offer a few advantages such as being able to sequentially sample tissue and fluids. The development of new animal models that more closely approximate human CMV disease is needed. This may include the murine cytomegalovirus (MCMV) infection as a model for drug resistance studies, as recent reports have demonstrated commonality between MCMV and HCMV DNA polymerase mutants. Only when such models exist can some of the questions relating to phenotypic characterization of resistant isolates be addressed.

Human studies which evaluate the stability and persistence of resistant CMV isolates also need to be performed. Data from such a study would determine whether patients who have a single episode of disease caused by a GCV-resistant isolate can ever again be treated with GCV. These trials should also evaluate the impact of prolonged oral GCV on the emergence of antiviral resistance. Clinical studies are also needed to evaluate the correlation of phenotypic and genotypic resistance with disease outcome in high-risk patient populations.

The susceptibility of a CMV isolate may vary according to the site from which it was isolated. This can be of clinical consequence, with detection of resistant virus correlating with disease progression. Such focal heterogeneity may be related to differences in turnover rates of virus in the different anatomic sites. Alternatively, the amount of drug available in such protected anatomic sites can be decreased compared to other areas of the body. Additional prospective human studies of such pharmacodynamics are urgently needed to optimize management of diseases such as CMV retinitis.

2.3. Varicella-zoster virus (VZV)

Mutations that confer ACV resistance among VZV isolates occur throughout the TK gene. Therefore, detection of the genotypic characteristics of resistant strains requires sequencing of the entire TK gene. This fact has direct implications for improved understanding of the process by which resistance develops in patients. Quantitative surveys of clinical subpopulations of VZV are needed to characterize the initial emergence of resistant variants and to monitor the rates at which susceptible isolates develop resistance.

The pathogenicity, virulence, and transmissibility of ACV-resistant VZV are largely unknown. Development of a good animal model in which the virus can be studied would provide a valuable tool by which such issues could be addressed. As with CMV, a SCID-hu mouse model is available but allows for only local replication of virus. As a result, the application of results to human disease has been called into question.

Clinical studies designed to improve understanding of the correlation between in vitro drug resistance and clinical outcome are also needed. Such investigations may help define a drug-susceptibility phenotype which can then guide therapy. Studies evaluating the role of combination antiviral therapy for VZV infections in selected immunodeficiency settings are also needed.

2.4. Influenza

Studies of amantadine and rimantadine in animal models have greatly enhanced the understanding of emergence of antiviral resistance and characteristics of resistant animal isolates; nevertheless, much work in humans remains. While both

rimantadine and amantadine offer significant benefits in the management of influenza disease, the rapid rate of emergence of antiviral resistance may limit their value. Human trials are needed to determine the frequency of emergence of resistance, and the clinical significance of drug-resistant influenza viruses. Such studies should determine whether resistant human influenza viruses are biologically stable. The ability of resistant isolates to compete with wild-type viruses for transmission in the absence of selective drug pressure requires further investigation. Furthermore, the degree of selective drug pressure necessary to cause substantial transmission of resistant viruses at the community level is not known; this could have serious implications from an epidemiologic viewpoint. If increased use should lead to identification of significant medical and epidemiologic problems attributable to resistance, practical ways for detecting and impeding emergence of resistance will be needed.

Despite the availability of effective vaccines, there remains a present and future need for effective antivirals for the prevention and treatment of infection and disease caused by the influenza viruses. Rimantadine and amantadine are effective only against influenza A viruses; there are no approved antivirals for influenza B virus infections. Aerosolized ribavirin has been shown to ameliorate the clinical course of both type A and type B infections, but it is not approved for use in influenza. There is promise for GG167, a new neuraminidase (NA) inhibitor, but clinical utility has yet to be shown. This inhibitor was developed through knowledge of the three-dimensional structure of the NA, information also available for the hemagglutinin (HA). The elucidation of the structure of the surface glycoproteins and the details of replication provide new opportunities for antiviral development for influenza.

Administration of rimantadine to animals infected with influenza A virus results in decreased lung virus titers, decreased cytotoxic T lymphocyte (CTL) responses, and decreased specific antibody responses. The decrease in CTL and antibody responses appears to be due to decreased amounts of viral antigen. The CTL response, interferon-α production, and other immune factors play a decisive role in promoting recovery from influenza virus infection. Further characterization of the human immune response to influenza infection, and the effect of antiviral therapy on this response, may lead to the development of additional strategies in the treatment of influenza disease. Such strategies might incorporate immune modulators into the antiviral regimen.

2.5. Picornaviruses

Some antiviral agents with activity against the picornavirus family have shown clinical efficacy in challenge studies. Analysis of these compounds, however, has been hindered by the lack of an animal model in which they can be studied. While the coxsackie virus B3 mutants can be evaluated in a mouse model, animal models

for other members of the picornavirus family do not exist. As such, the virulence of most of the mutant viral models is unknown. The development of additional animal models is a priority in this field.

2.6. Human immunodeficiency virus-1 (HIV-1)

The rapid advances occurring in the field of HIV research rival any development to ever occur in medicine. The amount of knowledge gained over the past fourteen years regarding the biology, epidemiology, and transmission of HIV is unparalleled. However, many areas exist in which substantial progress is required. The understanding of the mechanisms of HIV drug resistance at the cellular and enzymatic levels requires further development. Studies of how the baseline sequence (the "backbone") of the reverse transcriptase (RT) and protease may impact the expression of phenotypic drug resistance are also needed.

Standardization of assessment of phenotypic drug resistance requires the development of reference HIV isolates that are resistant to various antiretroviral agents. More rapid assays that screen for phenotypic HIV drug resistance are needed, as are alternative assays for the evaluation of resistance to the non-nucleoside RT inhibitors (NNRTIs) and the protease inhibitors (PIs).

With respect to genotypic drug resistance, development of methods to assess viral nucleotide sequences rapidly and reliably is needed. To standardize results from different laboratories, reference plasmids with known point mutations and combinations of mutations need to be developed and distributed. Defined mixtures of wild-type/mutated DNA and RNA would allow for assay validation between laboratories. Correlations between genotypic mutations and viral phenotype are also needed.

Adequate animal models of human HIV disease are lacking. While feline immunodeficiency virus (FIV) provides a potential model by which development of drug resistance can be evaluated, a highly pathogenic molecular clone of FIV is not available at this time. The simian immunodeficiency virus (SIV)/macaques model for human acquired immunodeficiency syndrome (AIDS) is promising for the study of primate lymphotropic lentiviruses. However, animal models that utilize the HIV-1 virus (the SCID-hu mouse, the chimpanzee, and the rabbit) have significant limitations in the evaluation of HIV disease. Development of additional animal models would provide a means by which new antiviral drugs could be tested and emergence of drug resistance assessed.

At the clinical level, the biologic properties of zidovudine (AZT)-resistant HIV isolates and their interactions with the host require clarification. Development of new and innovative treatment strategies must take into account focal heterogeneity and the degree of drug-virus interaction in protected sites. These strategies are likely going to increasingly rely on combination therapies to improve treatment efficacy, with assessment of response based upon evaluation of surrogate markers.

More data are needed to address the optimum choices of antiretroviral agents, and the sequence and combinations in which they should be used. Convergent combination therapies are among those that warrant further investigation.

Consensus Points and Recommendations

Molecular Mechanisms of Antiviral Resistance

Herpesviruses

- For HSV, resistance is conferred by mutations within the TK gene and the DNA polymerase gene.
  - TK-negative (TK): most common.
  - TK-partial (TKp).
  - TK-altered (TKa).
  - DNA polymerase-altered.
- For VZV, resistance is conferred by mutations within the TK gene and the DNA polymerase gene.
  - TK-negative (TK).
  - TK-altered (TKa).
  - DNA polymerase-altered: uncommon.
- For CMV, resistance is conferred by mutations within the UL97 phosphotransferase gene and the DNA polymerase gene.
  - 85% of GCV-resistant mutants have deletions or point mutations at one of three amino acid sites in the UL97 phosphotransferase.
  - DNA polymerase-altered.
- Amino acid changes that confer antiviral resistance are more conserved among CMV isolates than among HSV or VZV isolates.
- Mixed populations of resistant and sensitive viruses occur in clinical isolates for all the herpesviruses.
  - Affects the interpretation of assays of drug resistance.
  - Implications on the development of rapid assay systems that seek to detect degrees of resistance that are clinically significant.
  - Implications for the study of antiviral resistance in animal models.
  - Implications for the treatment of clinical disease.
- Need to better define the effect of genotypic changes on phenotypic expression.
  - Development of additional animal models will help in this area.

Influenza

- Mutations in the transmembrane M2 protein confer resistance to amantadine and rimantadine.
- M2 transmembrane mutations block the transport of ions into the virion core.
- Ion channel current measurements require physiologic conditions.
- Conditions which alter the α-helix structure of the M2 transmembrane domain warrant further investigation.

Picornaviruses

- At the present time, no therapeutic agents are licensed for the treatment of infection caused by any of the picornaviruses.
- Widespread clinical implications of the mechanisms of resistance are unclear.
- Resistance develops by multiple mechanisms.
  - High level resistance results from mutations to larger side chains in the drug-binding pocket, resulting in exclusion of drug from the pocket (rhinovirus 14 model, coxsackie virus B3 model).
  - Low level resistance results from mutations which increase the binding affinity between the virus and the host target cell (rhinovirus 14 model).
  - Stability mutants with drug-requiring phenotypes also have been described (rhinovirus 1A model).
- Resistant viruses appear to be attenuated in their virulence.
- Reports of the prevalence of resistant viruses varies by clinical study.
- Need additional models in which resistance can be studied, as well as additional clinical trials.

Human immunodeficiency virus-1

- Three classes of antiviral compounds:
  - Nucleoside analog inhibitors of viral reverse transcriptase (RT) enzyme (NRTI).
  - Non-nucleoside inhibitors of the viral RT enzyme (NNRTI).
  - Inhibitors of the viral protease (PI).
- Multitude of changes in amino acid sequences exist for each of these classes of drugs; in general:
  - For NRTIs, cross-resistance exists by nucleotide class.
  - NNRTIs share significant cross-resistance.
  - PI amino acid sequence changes result in cumulative resistance, especially at amino acids 82 and 84.

- The primary factor that mediates development of resistance is the extraordinarily and persistently high rates of viral replication which is unique to HIV.
- Need to better define the effect of genotypic changes on phenotypic expression.

Assays for Antiviral Drug Resistance

General

- Research assays are not used for decisions of clinical management of patients, and as such the time required to complete the assay is of less importance.
- Clinical assays, on the other hand, need to have a rapid turnaround time.
- Current phenotypic assays are dependent upon isolation, growth, and titering of viral isolates.
  - Requires measurement of infectivity both pre- and post-therapy.
  - Variability must be considered and evaluated for each test.
- Genotypic assays are performed directly from the in vivo specimen and do not require in vitro passage.

Herpesviruses

- While plaque reduction assays (PRAs) are relatively labor-intensive, they remain the method of choice for detecting resistance.
- PRAs can be effectively supplemented by:
  - DNA amplification techniques.
  - Direct antigen detection.
- Direct genotypic evaluation will be required to supplement whole-virus assays.
  - By correlating the genetic changes with the effect on viral TK production or function, an enhanced appreciation of expression of TK in the clinical setting will develop.
- Development of PCR-based rapid genotypic assays is most feasible for CMV.
- Serial monitoring of isolate susceptibilities is desirable.
  - Phenotypic changes can occur very rapidly.
    - Sensitive isolates become resistant.
    - Resistant isolates become sensitive.
- Correlation of laboratory resistance with clinical failure is strongest with HSV, and is weakest with VZV.
- Direct detection of resistant isolates may prove more clinically relevant due to elimination of viral expansion required for phenotypic assays.

Influenza

- The ELISA assay remains the gold standard for detection of antiviral resistance in influenza infection.
- Direct assays of the ion conductance properties of M2 and its pH-dependent activation characteristics may prove useful in detecting subtle changes in the activities of M2 mutants in research setting, but their applicability for mass screening is unknown.

Human immunodeficiency virus-1

- The standard assay is a PBMC colorimetric assay that requires four to six weeks to complete.
- Genotypic assessments have become routine but are complicated.
  - Combination antiviral therapy leads to the development of many combinations of mutations.
  - Mixed genotypes are difficult to detect.
- Selection bias will influence results of viral susceptibilities; such bias can be introduced by:
  - Cell line utilized.
  - In vitro passage and isolate expansion.
- Need to develop recombinant viruses to standardize susceptibility testing (reference isolates).
- Need to develop assays which screen for phenotypic drug resistance more quickly than the current PBMC-based assay system.
- Plasma quantitative RNA assays may provide better endpoints upon which changes in therapy can be based.
- Marker transfer experiments may lead to an enhanced understanding of the relative contributions of individual mutations on the overall resistance of an isolate.

Models of Antiviral Resistance

General

- A fundamental principal of all animal models is that they should mimic human disease as closely as possible.
- Animal models should use the human virus.
- Animal models should have pharmacodynamics which are similar to humans.
  - Differential metabolism of drug between humans and various animal models must be considered.

- The evaluation of mixed populations of virus in animal models can yield confusing results.

Herpesviruses

- Good animal models exist for HSV, but models for VZV and CMV are less relevant to human disease.
- Studies of resistant viruses document viral replication and disease.
  - However, the ability of the virus to reach the ganglia and to establish latency varies according to the model.
- Phenotypic characterization should include:
  - In vitro replication.
  - In vivo replication.
  - Replication in the target organ.
  - Lesion evaluation.
  - Latency and reactivation.
  - Mortality.
- Assessment of resistance must include a careful evaluation of viral genotype to better characterize the activities of low TK-producers (TKp) versus TK-negative (TK) strains in these model systems.

Influenza

- Good animal models exist, including:
  - Mice.
  - Ferrets.
  - Chickens.
- Assessment of resistance during antiviral therapy indicate:
  - Very rapid development of resistance.
  - Transmission of a genetically stable resistant virus.
  - The transmissible resistant virus retains its virulence.
- Consider development of combinations of therapeutic interventions, including:
  - Antiviral agents
  - Immune modulators
  - Vaccines

Human immunodeficiency virus-1

- At the present time, humans provide the only in vivo model for testing antiviral agents

  - This provides only a questionable system by which issues of resistance can be addressed.
- Adequate animal models of human HIV disease are lacking.
  - A highly pathogenic molecular FIV clone is not available at this time, limiting the utility of FIV in the evaluation of human HIV disease.
  - The simian immunodeficiency virus (SIV)/macaques model for human acquired immunodeficiency syndrome (AIDS) is promising for the study of primate lymphotropic lentiviruses.
  - However, animal models that utilize the HIV-1 virus (the SCID-hu mouse, the chimpanzee, and the rabbit) have significant limitations in the evaluation of HIV disease.
- Development of additional animal models is needed.

Antiviral Resistance in Clinical Practice

- During disease progression, one may or may not be able to isolate a sensitive virus, let alone a resistant one.
  - In the presence of progression of CMV retinitis, CMV often cannot be isolated by culture from a peripheral (non-ocular) site.
- Conversely, a resistant virus may or may not be causally associated with disease progression.
  - For HSV, disease progression in an immunocompromised host is usually associated with a resistant virus.
    - Thus, HSV offers the best example of a deterioration in clinical course reflecting a change in in vitro susceptibility.
  - With clinical failure of GCV in the management of CMV retinitis, use of foscarnet (PFA) can result in improvement in clinical course.
- From a management standpoint, empirical observation followed by a change in drug therapy has resulted in improved outcome.
- Drug resistance is only one variable in the progression of many clinical viral diseases.
  - HSV disease which progresses in the face of adequate ACV therapy will often respond to a change in antiviral agents to PFA.
  - CMV disease which progresses despite adequate GCV therapy will often respond to a change in antiviral agents to PFA.
  - In HIV disease, clinical failure and drug resistance are not identical.
    - Clinical failure can occur even when all HIV isolates continue to be sensitive to AZT.
    - Drug resistance is only one variable in the progression of clinical disease.
    - Other variables include CD4 cell count and plasma viral load.
- Resistant viruses are not by definition avirulent.
  - Resistant viruses can be transmissible.

    - HIV.
    - Influenza.
  - Resistant viruses can cause human disease.
    - HSV.
    - Influenza.
  - However, resistant viruses often are somewhat attenuated.
- Strategies to prevent emergence of antiviral resistance:
  - Avoid unnecessary drug exposure, especially for "at-risk" populations.
    - Recurrent genital HSV infection in HIV-positive patients does not automatically necessitate the use of suppressive ACV therapy.
  - Develop surrogate markers that are simple to perform and will indicate a progressive viral decrease in drug susceptibility.
- Treatment recommendations:
  - The drug of choice for the management of HSV and VZV infections is ACV; if resistance is suspected based upon in vitro susceptibilities or poor clinical response in immunocompromised patients, a change to PFA may be warranted.
  - The drugs of choice for the management of CMV infections are either GCV or PFA; if resistance is suspected based upon progression of disease or positive cultures despite adequate GCV therapy, a change to PFA may be warranted.
  - The drugs of choice for the treatment of influenza infection in selected individuals are amantadine and rimantadine.
  - Treatment for HIV infection is initiated with AZT; appropriate second line drugs and drug combinations for use at the time of disease progression are being evaluated in clinical trials at this time.

Special Recommendations

- Clinical surveillance should be enhanced.
  - Such surveillance should evaluate differences between viral isolates obtained from different body sites (focal heterogeneity).
  - Collection of surveillance isolates should also be incorporated into ongoing prospective clinical trials.
  - Prevalence of resistance and transmission of resistant isolates can be monitored.
- More thorough correlation of disease outcome with markers of resistance is needed.
- Additional rapid assays should be developed for application in human clinical trials.
- The availability of assays to detect emergence of resistance during clinical trials is an essential component of the development and distribution of a new drug.

- Improved management strategies need to be formulated.
  - Development of new antiviral drugs will lead to development of innovative treatment strategies.
- Development of genotypic and phenotypic reference standards is needed for all the viruses discussed in this symposium, but especially for the herpesviruses and HIV.
- A central repository needs to be established, with stocks of standardized reference viruses, reference reagents, and enzymes available upon request to investigators.
- Distribution of new chemical compounds to individual investigators for research purposes should be encouraged.
- A working group of experts in the field should be convened for the purpose of standardization of nomenclature and terminology.
  - Use of "μg" versus "μM".
  - Use of "IC_{50}" versus "EC_{50}".
  - Define breakpoints above which clinical failures are likely to occur.
    - Values for resistance by HSV-1, HSV-2, CMV, and VZV to the five licensed (or soon to be licensed) antiviral agents (ACV, GCV, BV-ara-U, PCV, PFA) should be determined.