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REVIEW

Virological and Immunological Outcomes of Coinfections

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SUMMARY Coinfections involving viruses are being recognized to influence the disease pattern that occurs relative to that with single infection. Classically, we usually think of a clinical syndrome as the consequence of infection by a single virus that is isolated from clinical specimens. However, this biased laboratory approach omits detection of additional agents that could be contributing to the clinical outcome, including novel agents not usually considered pathogens. The presence of an additional agent may also interfere with the targeted isolation of a known virus. Viral interference, a phenomenon where one virus competitively suppresses replication of other coinfecting viruses, is the most common outcome of viral coinfections. In addition, coinfections can modulate virus virulence and cell death, thereby altering disease severity and epidemiology. Immunity to primary virus infection can also modulate immune responses to subsequent secondary infections. In this review, various virological mechanisms that determine viral persistence/exclusion during coinfections are discussed, and insights into the isolation/detection of multiple viruses are provided. We also discuss features of heterologous infections that impact the pattern of immune responsiveness that develops.

KEYWORDS bystander protection, diverse TCR repertoire, attrition, coinfection, cross reactivity, exclusion, persistence, virus

INTRODUCTION

It is common to attribute a viral disease to infection by a single agent. However, under natural circumstances hosts may be infected by multiple agents with the outcome influenced by contributions from more than the incriminated virus, but rarely in diagnostic laboratories do we consider the input of multiple agents. Regarding terminology, infection by more than one variety of microorganism (viruses, bacteria, protozoa, etc.) is termed mixed infection. In virology, coinfection is used to describe simultaneous infection of a cell or organism by separate viruses (1). The term superinfection is used if one virus infects the host some time before infection by the second virus. However, in the literature, the definitions of coinfection and mixed infection have been used interchangeably (2–5). The meaning of these terms depends on the context, whether applied to a single cell, a cell line, part of a host, or a whole host (1, 4). In an infected cell, viruses can interact with a large number of cellular proteins (virus-host interactome) that may either support or inhibit virus replication. As with virus-host protein interactions, protein-protein interactions between unrelated viruses are also possible (6, 7). Coinfections may result in genetic exchange between agents to generate recombinant viruses. Chimeric viruses (mixed nucleic acid) observed in metagenomic studies have suggested the possibility of genetic exchange even among heterologous viruses, but this issue needs further evaluation (8). Recombination effects can influence viral evolution, disease dynamics, sensitivity to antiviral therapy, and eventually the fate of the host (9).

Coinfections may play a pivotal role in reducing or augmenting disease severity (10–13). However, because of the high specificity of diagnostic assays, they usually miss

detection of additional relevant agents. When individual cells are coinfected, one virus usually influences replication of the other, a phenomenon termed viral interference. The result can be clearance (exclusion) of one virus but persistence of the other (14). Viral interference may be mediated by factors such as interferons (IFNs), defective interfering (DI) particles, production of *trans*-acting proteases, cellular factors, and nonspecific double-stranded RNA (dsRNA) (1). Besides virus-virus interactions, the nature of the host also plays an important role in shaping coinfection patterns. For example, bacterial isolates from a particular geographical region are usually infected more efficiently by bacteriophages isolated from the same niche (15).

The response of the host immune system also influences the outcome of viral coinfections. Upon antigen exposure, naive T cells convert into activated effector T cells and eventually long-term memory T cells. Memory responses generated against one infection may influence the quantity and quality of the immune response to subsequent secondary infection. This influence of immunity to primary infection on a subsequent unrelated infection is known as heterologous immunity. Heterologous immunity can occur between very closely related infectious agents such as multiple variants of a particular virus type, among different viruses, or between viruses, bacteria, protozoa, or different parasites (2). A variety of immune cells participate in heterologous immunity, and these may induce either a protective or immunopathological response (2). Finally, studying coinfections in short-lived laboratory animal systems can be misleading since the outcome of coinfections in clean containment facilities does not replicate what occurs in natural environments in hosts exposed often for decades to multiple pathogens.

DETECTION OF COINFECTIONS

Multiple viruses are capable of causing disease syndromes, though we usually consider the outcome of infection by a single virus. However, almost invariably under natural circumstances, hosts may be infected by multiple agents, with the outcome influenced by contributions from more than a single agent. In diagnostic laboratories, we rarely consider the input of multiple-agent infections. Current understanding of mixed infections is biased and is targeted on the culturable or presumed disease-causing agents. The laboratory investigation of disease is usually directed to correlate the clinical symptoms with a particular pathogen, with the aim of establishing that agent as the etiology. In reality, the disease could be associated with multiple agents. Therefore, the clinical implication, diagnosis, and therapeutic management of such viral infections are of considerable importance. Unlike bacteria, where individual organisms can be rapidly purified from a mixed culture by colony purification, multiple viruses cannot be easily purified directly from clinical specimens. For virus isolation, the clinical specimens need to be detected in an appropriate host; this approach permits amplification of the divergent viruses present in the clinical specimens. Unfortunately, divergent viruses in a specimen may block replication of the target virus (viral interference) and hence result in a misdiagnosis. Classically, the detection of the coinfection has been based on serology and virus isolation, both of which may be compromised by inadequate sensitivity and specificity. The advent of PCR in the 1990s enhanced the specificity and sensitivity of coinfection detection, but because PCR amplification needs prior sequence information on the target genome, PCR encounters problems when amplifying for divergent viruses from clinical specimens. Next-generation sequencing (NGS) platforms have completely revolutionized virus diagnostics and novel virus discovery. NGS does not need prior sequence information about the target genome and allows detection of most potential genomes present in the clinical specimens, and therefore it is considered highly effective for the detection of multiple agents (16–19). However, isolating multiple viruses in a purified form is cumbersome and is rarely achieved. Viruses have variable host range/tropism. Consequently, in a particular cell type, one virus usually replicates faster, eventually resulting in the elimination of other coinfecting viruses upon long-term culture.

Virus Isolation

Compared to the use of embryonated eggs and laboratory animals, employment of

TABLE 1 Strategies for purification of multiple viruses from mixed culture

Strategy	Remark
Treatment with organic solvents to eliminate enveloped viruses	Unsuccessful if the concn of the organic solvents required for complete inactivation of the virus particles is toxic to the target cells
Removal of hemagglutinating viruses	Complete adsorption of hemagglutinating virus is difficult to achieve
Plaque assay	Not all viruses form plaques
Limiting-dilution assay	Quite cumbersome, as testing so many replicates by PCR is labor-intensive
Neutralization with antiserum	Considered an ideal strategy for targeted elimination of a known virus; however, at lower passage levels, virus may not form CPE, and at higher passage, when it starts forming CPE, defective interfering particles may appear that interfere with plaque formation as well as facilitate extinction of standard viral genome
Passage in cell types that do not support growth of divergent viruses	Depends on virus(es) and cell types used for coinfection
Treatment with acid/alkali	One of the coinfecting viruses may be sensitive toward extreme pH; therefore, it can be eliminated by exposure to extreme pH
Viral RNA transfection	Most efficient method for the elimination of RNA viruses with negative-sense genomes

cell culture in laboratories in the 1960s provided a less expensive and more convenient tool for virus isolation. Besides the diagnostic utility, virus isolation is essential for product development (vaccines and diagnostic agents) and is also crucial for clinical decisions such as discriminating disease from subclinical infections (20) and deciding when to implement, change, continue, or discontinue drug therapy (21). Isolating multiple viruses in a purified form represents a major bottleneck in cases of coinfections. The presence of a viral genome or antigen in a clinical specimen does not always warrant virus isolation (22, 23). During cell culture adaptation of a virus (virus isolation), several blind passages are usually required before appearance of cytopathic effects (CPE) (24). It is likely that due to a difference in the rates of replication or due to viral interference, one of the viruses will be eliminated before appearance of CPE. If the culture conditions are more permissible for the adventitious virus, it is likely that it will exclude the targeted agent on high passage, thereby resulting in failure of the targeted isolation of a known virus. Even under conditions where both the coinfecting viruses are able to persist until the appearance of CPE, it is not mandatory that both of them will participate in the formation of CPE (14). However, in such instances, at least one of the viruses can be purified by plaque assay (14). Moreover, we have witnessed conditions where despite formation of CPE (in mixed culture), none of the coinfecting viruses formed plaques (14), though subsequent higher passage of the mixed culture allowed plaque formation by one of the viruses (14).

Depending on the nature of coinfecting viruses, strategies for virus purification from mixed culture vary (Table 1) and may include (i) elimination of the enveloped viruses by treatment with the organic solvents (25), (ii) hemagglutination to separate a hemagglutinating virus, (iii) endpoint dilution assay to purify multiple agents, (iv) antibody (Ab) neutralization to eliminate other confecting viruses (26), (v) acid/alkali treatment if one of the viruses is more susceptible to extreme pH, (vi) plaque assay to purify single or multiple viruses, and (vii) transfection of the viral RNA mixture into target cells, which allows amplification (production) of only positive-sense RNA viruses, thereby eliminating negative-sense RNA viruses from the mixed culture (14).

Complications in Isolation of Multiple Viral Agents

Inability to produce CPE. Isolation/purification of multiple viral agents from natural infection is quite cumbersome (Table 1). In the beginning of cell culture adaptation, viruses usually do not show cytopathic effects (CPE) (are noncytolytic), and so plaque purification is not feasible. Later, when CPE is evident, all coinfecting viruses may not contribute to CPE formation, thereby allowing purification of only CPE-forming virus. Under such circumstances, antibody neutralization of the cytolytic virus may allow purification of the noncytolytic viruses. However, further blind passages may be required until noncytolytic virus does become cytolytic (14).

DI particles. Defective interfering (DI) particles are produced following high-multiplicity-of-infection (high-MOI) passage of a virus in cell culture (27, 28). DI particles

have a defective or deleted genome, replicate quite rapidly compared to the wild-type (WT) virus, and generally require another helper virus (wild type) for effective replication (29–31). Two defective RNA genomes may also act synergistically to produce cytopathology (32). DI particles may hamper the plaque-forming ability of WT virus (14, 33). The presence of DI particles progressively reduces levels of standard viral genome such that at higher passage levels, the wild-type viral genome may not be detectable by PCR (14, 32, 34, 35). DI particles also produce rapid CPE, and this may prematurely terminate the life cycles of other coinfecting (homo- or heterologous) viruses, eventually resulting in their extinction. However, little is known about direct interaction of DI particle with a heterologous virus.

Rescue of positive-sense RNA virus directly from clinical specimens. Viruses with positive-sense RNA genomes can generate infectious virus upon delivery of their viral RNA into host cells. This property may be exploited to eliminate negative-sense RNA viruses from mixed cultures. However, in most instances, viral RNA derived only from the cell culture-adapted viruses, but not that from clinical specimens, produces CPE in the established cell lines (14). Transfecting viral RNA (derived from clinical specimens) into primary cells may sometimes show rapid CPE (36, 37), although the reduced amount of viral RNA may require additional passages until CPE becomes observable in primary cells (36). The RNA delivery method, which allows elimination of the DI genome (14), is considered more suitable than antiserum treatment for purification of positive-sense RNA viruses from mixed culture (Table 1).

Improved Virus Isolation

The selection of appropriate body sites and the proper collection, transport, processing, and preservation (freezing conditions) of specimens all contribute to enhance the success of virus isolation. Specimens with large amounts of virus (24, 38) and centrifugation-enhanced inoculation also increase the chances of isolating viruses from clinical specimens (39). A single cell line is not always suitable for isolating multiple viruses, but cocultured and genetically modified cell lines have made it possible to simultaneously isolate multiple viruses.

Cocultured cells. As a consequence of isolation in cell culture, viruses may undergo genetic changes (40). The success of virus isolation may also depend on the nature of cells used for infection, and a single cell type may not always be appropriate for isolation of multiple viral agents (5, 14, 41). Cocultured cells, where multiple cell types are cultured together in a single monolayer, may solve the problem of isolating multiple viruses (42, 43), and to this end, a variety of mixed cell cultures have been recommended for detection/isolation of multiple viruses. A mixture of MRC-5 and A549 cells is useful to detect cytomegalovirus (CMV), herpes simplex virus (HSV), and adenovirus in the same specimen and can be as sensitive as immunofluorescence or isolation in a single cell type (43). Similarly, a coculture of mink lung and human adenocarcinoma cells (R-Mix cells) is useful for the rapid isolation of respiratory viruses (parainfluenza virus types 1, 2, and 3, influenza A and B viruses, rouse sarcoma virus [RSV], adenovirus, HSV, CMV and enteroviruses) (44–48). R-Mix cells also facilitate the isolation of highly pathogenic respiratory viruses such as severe acute respiratory syndrome coronavirus (SARS-CoV), which cannot be grown without a containment laboratory. Therefore, there might be a risk associated with use of R-Mix cells for virus isolation. An alternative approach being used is the R-MixToo cell line (consisting of MDCK and A549 cells), which does not support SARS-CoV infection (49) and is more sensitive than R-Mix cells for detection of influenza B viruses and adenovirus (50). Both R-Mix and R-MixToo cells facilitate growth of diverse strains of influenza viruses (51, 52) and provide a faster and sensitive cell culture system for isolation of respiratory viral agents. The times needed for positive cultures are 1.4 and 5.2 days, respectively, for R-Mix and single culture (46, 52). Additionally, a mixture of MRC-5 and CV1 cells facilitates multiplex detection of HSV-1, HSV-2, and varicella-zoster virus **(**VZV) (53, 54). The CPE formed in these cocultured cell lines is as sensitive as fluorescence-based assays (54). Finally, Vero/BHK-21 cocultured cells are adequate for concurrent isolation

of peste des petits ruminants virus (PPRV) and foot-and-mouth disease virus (FMDV) (14). These cocultured cell lines are also quite sensitive for the detection/isolation of viral agents with a very low virus titer and those which grow slowly (42). However, their cost is usually higher than that of a single-cell culture (42).

Transgenic cell lines. Some genetically engineered cell lines (transgenic cell lines) have been developed to enhance the efficiency of virus detection (41, 55–58). A genetically modified cell line named BHKICP6lacZ-5 (enzyme-linked virus-inducible system [trade name ELVIS]; Diagnostics Hybrids, Inc.) which uses an HSV promoter sequence (UL39 gene) in association with *Escherichia coli lacZ* was developed. Within a few hours of HSV-1/HSV-2 infection, virus-associated transactivators strongly activate the promoter (55) to induce β-galactosidase that can be detected with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (a chromogenic substrate) (55). Whereas single-cell systems detect virus (CPE) in 48 h, BHKICP6 transgenic cell lines can detect virus within 16 to 24 h (59). The original ELVIS approach detected only HSV, but it has now been modified to distinguish HSV-1 and HSV-2 (42, 60, 61) and is less expensive but less specific than PCR (42).

The field needs a cell line system to detect multiple enterovirus strains. Human embryonic lung fibroblasts and primary monkey kidney, A549, and BGMK cells are generally used for enterovirus isolation, and these produce CPE within 5 days (62). Compared to use of a single cell type, coculturing these cells has enhanced the possibility of virus isolation (63, 64). Compared with wild-type BGMK cells, BGMK-hDAF, a genetically engineered cell line expressing human decay-accelerating factor (hDAF) and with an expanded host range, can enhance enterovirus detection (64, 65). The sensitivity of these cell lines was further increased by coculturing BGMK-hDAF with CaCo-2 (BGMK-hDAF/CaCo-2 [marketed as Super E-Mix cell; Diagnostic Hybrid Inc.]) (65).

Hemadsorption. Hemadsorption is useful approach to detect viruses which produce slow or no CPE in cultured cells (5, 42). Hemadsorption is applicable to those viruses that express hemagglutinin proteins on the plasma membrane of infected cells. Examples include members of the family *Orthomyxoviridae* and *Paramyxoviridae*. Hemagglutination testing is usually performed in virus-infected cells by replacing cell culture medium with a suspension of red blood cells. Hemadsorbing foci can be seen as early as 12 h following infection with influenza A and B viruses (39).

Nucleic Acid-Based Tests

Multiplex PCR. PCR directly targets viral genomes and is more specific than enzyme immunoassays. However, PCR is labor-intensive and expensive, particularly for the detection of multiple viral agents. Quantitative real-time PCR (qRT-PCR) for concurrent detection of heterogeneous viruses in a single reaction has reduced the overall cost (66–81). Although the qRT-PCR system is quite sensitive, adsorption and fluorescence spectra for different fluorophores used in the fluorescent-labeled probe systems tend to interfere with each other, which limits reliable detection to a maximum 4 or 5 different viruses (82).

Chemiluminescence and magnetic separation. Detection of a limited number of fluorophores, which is a drawback of qRT-PCR, may be overcome by employing a chemiluminescent label-based assay (83, 84). Optical labels such as colorimetric nanoparticles (85–87), fluorescent tags (88), and chemiluminescent labels (89) are increasingly being used for DNA hybridization assays. However, due to simple instrumentation, increased sensitivity, and low background, chemiluminescent label-based techniques are preferred over fluorescence-based detection (90, 91). Because of their easy manipulation under an external magnetic field, surface-modified magnetic particles can be used for enrichment of the target molecules, and this permits high-throughput and automated detection platforms (92–95). Based on these advancements, Ali et al. combined magnetic separation technology (for nucleic acid purification) with a chemiluminescence technique for more sensitive (as low as 10 viral RNA copies) detection of multiple viral agents (82). The technique involved simultaneous extraction of the viral nucleic acid and amplification of the viral genomes in a single tube by qRT-PCR (with

biotin-11-dUTP being incorporated into the amplified products during amplification). This was followed by capture of the virus-specific gene segments by different amino-modified probes attached with carboxyl-coated magnetic nanoparticles (82).

NGS. Despite the availability of a wide range of sensitive and specific diagnostic assays, profiling of microbial species has not been possible. Microarray-based methods, such as ViroChip (96–100) and PathChip (101, 102), that allowed detection of multiple agents but did not support detection of a divergent virus were developed. Sequence-independent amplification techniques (next-generation sequencing [NGS] platforms) have been successfully employed for rapid detection of novel (16–19) and multiple (103) viruses in clinical settings (104–106) and have allowed whole-virus genome organization (107) and analyses of minority variants (108, 109). NGS detects sequences from almost all potential organisms in an unbiased manner. It also allows concurrent genetic characterization of diverse groups of known viruses as well as divergent viruses that evade conventional testing (110, 111). For example, transcriptome analyses of >220 invertebrates identified 1,445 RNA viruses, including those that represented new virus families (112).

Conventional NGS systems omit detection of single-stranded DNA (ssDNA) viruses, although modified library preparation has now made it possible to amplify/detect ssDNA viruses (113–117). The main disadvantage of NGS is the high cost and unsuitability for high-throughput application to detect viruses in multiple clinical samples. Moreover, guidelines that allow interpretation of viral sequences with clinical relevance are lacking (103). In a given clinical specimen, NGS reveals both viral and cellular sequences, but patient privacy must be maintained before transmitting the data from research into clinic settings (103). Nevertheless, the cost of NGS is sharply declining, and in the future it may be competitive with current diagnostic assays (107).

ViroCap (probe enrichment). NGS often fails to detect viruses detectable by PCR (118) and may not produce sufficient data for comprehensive analysis of the viral genomes, particularly in specimens that contain minimal virus. Several strategies can be used to increase the virus-specific sequence reads. For example, low-speed centrifugation and filtration to remove host/bacterial cells, treatment with nucleases to remove free nucleic acids (not encapsidated by virus), and ultracentrifugation to increase the concentration of virus particles improve the approach. However, employment of these enrichment strategies is not sufficient to capture all viral sequences present in clinical specimens.

ViroCap is a test system developed recently (119) that is based on a targeted sequence panel to enrich viral genomes and includes 190 viral genera and 337 species. To define a unique set of reference sequences, ~1 billion bp of annotated viral genome sequences was reduced to <200 million bp of targets. This probe enrichment process involves hybridization of DNA/RNA probes to the cDNA fragments in a shotgun library. This is followed by 10 to 15 cycles of PCR prior to sequencing. Besides comprehensively detecting most vertebrate viruses, this system can detect divergent viruses having low sequence similarity (~50%) to the known vertebrate viruses (119). Compared to NGS, ViroCap increases virus detection by >50%. Because the targeted sequence enrichment increases the percentage of virus-associated sequence reads, it yields better viral coverage and needs fewer of total sequence reads. ViroCap has the potential to reduce sequencing cost and is flexible, since new viral sequences may be periodically added to increase representation of viruses in the shotgun library. However, ViroCap is incapable of detecting novel viruses (that do not share any nucleotide sequence similarity with known viruses), and the technology is still in the validation phase. It may take a few years until it is available for clinical use.

Heteroduplex mobility analysis (HMA). If multiple strains/subtypes of a virus are present in a clinical specimen, PCR amplification results in two heterologous double-stranded DNA products of similar size. When these heterologous DNA fragments are denatured and allowed to anneal, they form homo- and heteroduplexes, which are derived from identical and nonidentical strains, respectively. The formation of these homo- and heteroduplexes (nucleotide mismatches) results in altered migration in

agarose gel electrophoresis. This method has been utilized to illustrate divergent sequences present in torque teno virus (TTV) and hepatitis C virus (HCV) (120). Likewise, amino acid alterations in cytopathic and noncytopathic form of bovine viral diarrhea virus (BVDV) could be analyzed by distinct polypeptide profiles in virus-infected cells (121).

Multicolor Imaging with Self-Assembled Quantum Dot Probes

Multicolor quantum dot (QD) probes allow simultaneous detection and evaluation of coinfection of a cell by multiple viral agents. The process involves conjugation of quantum dot probes with *Staphylococcus aureus* protein A (SpA) and virus-specific antibodies (Abs). The application of a cocktail of multicolored QD-SpA-Ab probes to coinfected cells generates multiple fluorescence. This method has allowed simultaneous detection of influenza A virus (IAV) subtypes H1N1, H3N2, and H9N2 and human adenovirus in coinfected cells (122).

Laboratory Viral Stocks Contaminated with Unknown Viruses

Unlike for bacteria, where mixed cultures can be rapidly purified by plating on agar, virus purification from mixed culture remains a challenge. Whereas some of the viruses may be plaque purified, those which do not form CPE are cumbersome to purify. The clinical specimens may also contain cryptic viral agents. If the cell line is equally susceptible and the life cycle of the cryptic agent is shorter, the target virus is likely to be eliminated (viral interference) after few passages, even before its adaptation (CPE formation) in the cell culture system. Such divergent viruses may also be acquired accidently during *in vitro* propagation of the clinical specimens, although their presence is difficult to realize unless examined. Our laboratory is part of a culture collection center (repository). We faced such a problem when a parvovirus isolate came to our repository for deposition. We authenticated the virus deposit by observing CPE in MDCK cells and amplification of parvovirus-specific genome by PCR, and thereafter an accession number was assigned. Four years later, the virus isolate was distributed to another laboratory, where it was grown in A72 cells. After a few passages, the culture was found to be negative for the parvovirus genome. Upon further investigation, it was found to be positive for canine adenovirus. When the original virus stock which came to us for deposition was examined, it was found to be positive for both parvovirus and adenovirus, suggesting coinfection of these viruses in the original culture. The A72 cells favored the growth of adenovirus over parvovirus, and the latter was eventually eliminated. It is not possible to detect such divergent (unknown) viruses by virus species-specific assays, although NGS has made it possible to detect most potential genomes (pathogen/host) in clinical specimens (16, 123).

VIROLOGICAL OUTCOMES OF COINFECTIONS

Coinfections are increasingly being reported (Table 2). However, little is known about their effect on other coinfecting agents and the host. The most common outcome of coinfection is viral interference, where one virus competitively suppresses replication of the other confecting viruses. Besides interference, coinfections of certain viruses may also promote an increase in viral replication. In several other cases, coinfections have no effect on virus replication, and thus all the coinfecting viruses can coexist (accommodation). Coinfections are generally believed to exert a negative effect on health (124). They may modulate viral virulence and cell death, thereby altering disease severity and epidemiology. Establishing the outcome of coinfections requires integrated monitoring and research on multiple pathogens. However, there is a dearth of such data.

Viral Interference (Competitive Suppression)

A phenomenon whereby one virus interferes with the replication of other viruses so as to become resistant towards a second superinfecting virus is termed viral interference (1). Innate viral interference mediated via interferons (IFNs) is the most common form of viral interference (125, 126). Upon binding with their cognate receptors, IFNs

TABLE 2 Viral coinfections, detection, and outcomes[a]

Confecting viruses	Outcome	Method(s) of detection	Remark(s)	Reference(s)
DNV and CHIKV	Accommodation	Nucleic acid	CHIKV neither triggered nor suppressed DNV replication; mosquitoes with DNV infection were equally susceptible to infection by CHIKV	145
DNV and DENV	Interference/ enhancement	Nucleic acid	Reduced DENV replication concomitant with increased DNV replication	146, 147
DNV and DENV	Accommodation	Nucleic acid	Persistent DENV and DNV coinfection	148
DNV, DENV, and JEV	Accommodation	Immunofluorescence	Stable infection of all three viruses without any CPE	149
CHIKV and JEV	NA	Antibody	Prevalence of antibodies against dual infection	150
IBV and avian pneumovirus	Interference	Viral titers, nucleic acid, antibody	IBV interfered with replication of pneumovirus vaccine strain in fowl	151
IBV and NDV	Interference	Viral titers, nucleic acid	IBV interfered with NDV replication	152–162
Sylvatic and endemic DENV strains	Interference	Viral titers	Primary virus suppressed secondary virus infection	141
NDV and HPAIV	Interference	Viral titers	NDV blocked HPAIV replication	163
WMV and ZYMV	Interference	Nucleic acid	ZYMV inhibited WMV replication	164
Henipavirus and rubulavirus	NA	Nucleic acid	Evidence of dual virus infection in bats	165
SINV and LACV	Interference	Viral titers	Replication of both viruses suppressed	166
SINV and LACV	Interference	Viral titers	LACV titers suppressed but no effect on SINV titers if SINV infection was 2 h before LACV infection	166
DENV2 and DENV4	Interference	Viral titers	Suppression of both viruses but greater suppression of DENV2	142
SINV, SFV and SINV, RRV	Exclusion	Viral titers	Persistently SINV-infected cells excluded superinfecting heterologous alphaviruses	167
SINV and YFV	Accommodation	Viral titers	Persistently SINV-infected cells did not impair YFV replication	167
CxFV and WNV	Enhancement	Viral titers/nucleic acid	Enhanced WNV transmission in mosquitoes	168
CxFV and WNV	Accommodation	Viral titers/nucleic acid	CxFV had no impact on WNV replication	168
IPNV and VHSV	Interference/ accommodation	mRNA	Accommodation on coinfection and primary VHSV and secondary IPNV infection but interference on primary IPNV and secondary VHSV infection	169
NDV and LPAIV	Interference	Viral titers	Coinfection decreased LPAIV shedding and transmission but had no impact on clinical signs.	170
NDV and HPAIV	Interference	Viral titers	Coinfected ducks survived for shorter duration	170
HSV and VZV	Exclusion	Immunofluorescence	Exclusion of each other	171
BHV-1 gD and HSV-1/BHV-1/PRV	Interference	Viral titers	Expression of BHV-1 gD inhibited HSV-1, PRV, and BHV-1 replication	172
TTSuV1a and ASFV	NA	Nucleic acid	NA	173
hMPV and hRSV	NA	NA	Increased hospitalization rates in humans	174
HCV and TTV	NA	Nucleic acid (HMA)	NA	120
Fowlpox virus and ILTV	NA	Viral titers/nucleic acid	NA	175
WSSV and IIHNV	NA	Nucleic acid/ histopathology	Increased mortality in Pacific white shrimp	176
Influenza A/H1N1 and A/H3N2 viruses	NA	Nucleotide sequencing	Demonstrated ability of these two influenza virus subtypes to coinfect humans and a potential risk of influenza virus reassortment	177
CIAV and IBDV	Interference/ enhancement	Nucleic acid/cytofluorometric analysis	Enhanced CIAV titers in bursa and thymus but diminished IBDV-induced lymphocyte disorder	178
Cytopathic and noncytopathic BVDV	NA	Radioimmunoprecipitation/ polypeptide profile	Induced different polypeptide profiles	121
Multiple coronaviruses (BtCoV HKU2, BtCoV HKU8, BtCoV HKU1, BtCoV HKU7, BtCoV HKU10)	NA	Nucleotide sequencing	Evidence of multiple coronavirus infections in bats (zoonoses)	179
SINV and DENV4	Interference	Viral titers/nucleotide sequencing	SINV infection resisted DENV infection	180
ZIKV, CHIKV, and DENV	NA	Nucleic acid/antibody	Cross-reactivated antibodies against ZIKV/DENV may lead to misleading serological conclusions	181

(Continued on next page)

TABLE 2 (Continued)

Confecting viruses	Outcome	Method(s) of detection	Remark(s)	Reference(s)
CHIKV and DENV	NA	Nucleic acid/antibody	CHIKV and DNV were successfully purified by plaque purification and antibody neutralization, respectively	26, 182–187
DENV3 and CHIKV	Interference	Cell culture/nucleic acid	Interference depended on virus dose	143
DENV and CHIKV	NA	Antibody	NA	188
DENV1 and DENV3	Interference	Antibody	Higher DENV3 prevalence	189
GPV and ORFV	NA	Nucleic acid	NA	190
Human adenovirus, human enterovirus, RSV, and human rhinovirus	NA	Nucleic acid	NA	191
HIV-1 and influenza virus	Enhancement	NA	High risk of influenza infection in HIV-1-infected individuals	192
PCV2 and CSFV	NA	NA	Induction of stress response and apoptotic signaling pathways	193
DENV and HIV-1	NA	Nucleic acid	NA	194
DENV, CHIKV, and ZIKV	NA	Nucleic acid	Concurrent circulation of DENV, CHIKV, and ZIKV and their coinfection	195
PPRV and FMDV	Interference	Viral titers/nucleic acid	Reciprocal replicative suppression in BHK-21 and Vero cells, respectively	14
Aura virus, SFV, RRV, and flaviviruses	Interference	Viral titers/nucleic acid	Insect cells persistently infected with SINV resisted infection of both homologous (SINV) and heterologous (Aura virus, SFV, and RRV) alphaviruses but had no effect on flaviviruses	167
IAV and RSV	Interference	Viral titers/ immunofluorescence	IAV competitively suppressed RSV at the level of viral protein synthesis and budding	196
IAV and hPIV2	Enhancement	Viral titers/ immunofluorescence	hPIV2 facilitated IAV replication	197
Wild-type and oseltamivir-resistant IAV strains	Interference	Viral titers/nucleotide sequencing	H3N2 and H1N1 differed in their ability to suppress replication and in transmissibility of the respective drug-resistant viral mutants	198
Swine influenza virus and PRRV	Interference	Viral titers/nucleic acid	Primary virus infection interfered with replication of the secondary virus	199
Group 1 and group 2 Brazilian vaccinia viruses	Interference/ enhancement	Viral titers/nucleic acid	Higher titers in lungs, lower titers in spleen; greater disease severity in mice	200
HBV and HCV	Coexistence	Viral titers/nucleic acid	Coexistence in Huh-7 cells without any interference	201
WNV (different strains)	Interference	Viral titers	Block in transmission of superinfecting virus	202
WNV. SLEV	Interference	Viral titers/nucleic acid	Block in transmission of superinfecting virus	203
HBV and HCV	Enhanced disease severity	Antibody	HBV-exposed individuals experienced enhanced HCV-associated disease severity	204

[a]Abbreviations: ASFV, African swine fever virus; BHV, bovine herpesvirus; BVDV, bovine viral diarrhea virus; CHIKV, chikungunya virus; CIAV, chicken infectious anemia virus; CSFV, classical swine fever virus; CxFV, culex flavivirus; DENV, dengue virus; DNV, densovirus; FMDV, foot-and-mouth disease virus; GPV, goatpox virus; HCV, hepatitis virus; HDA, heteroduplex mobility analysis; HIV-1, human immunodeficiency virus type 1; HMA, heteroduplex mobility analysis; hMPV, human metapneumovirus; HPAIV, highly pathogenic avian influenza virus; hRSV, human respiratory syncytial virus; HPIV, human parainfluenza virus; HSV, herpes simplex virus; IBDV, infectious bursal disease virus; IBV, infectious bronchitis virus; IIHNV, infectious hypodermal and hematopoietic necrosis virus; ILTV, infectious laryngotracheitis virus; IAV, influenza A virus; IPNV, infectious pancreatic necrosis virus; JEV, Japanese encephalitis virus; LACV, La Crosse virus; LPAIV, low-pathogenic avian influenza virus; NDV, Newcastle disease virus; ORFV, Orf virus; PCV, porcine circo virus; PIV, parainfluenza virus; PPRV, peste des petits ruminants virus; PRV, pseudorabies virus; RRV, Ross River virus; RSV, respiratory syncytial virus; SFV, Semliki Forest virus; SINV, Sindbis virus; SLEV, St. Louis encephalitis virus; TTSuV1a, torque teno sus virus strain 1; TTV, TT virus; VHSV, viral hemorrhagic septicemia virus; VZV, varicella-zoster virus; WMV, watermelon mosaic virus; WNV, West Nile virus; WSSV, white spot syndrome virus; YFV, yellow fever virus; ZIKV, Zika virus; ZYMV, zucchini yellow mosaic virus; NA, not applicable.

induce multiple so-called interferon-stimulated genes (ISGs), many of which activate numerous cell signaling pathways (127–137). These ISGs regulate the activity of numerous innate immune mediators that nonspecifically block virus replication.

Non-interferon-mediated viral interference, also called intrinsic interference, is a virus-induced cellular state of resistance to subsequent viral infection. Initially it was observed in Newcastle disease virus (NDV) superinfection where the refractory state against NDV emerged exclusively in cells that experienced prior viral infection. The effect was due to molecules encoded by the virus (viral genome/proteins) and not to the intrinsic capacity of cells (138). Later this was also observed in FMDV, where the attenuated A24 Cruzeiro strain interfered with the multiplication of a homologous

TABLE 3 Mediators of viral interference

Mediator(s)	Remark(s) or virus(es) involved	Reference(s)
Defective interfering particles	FMDV	14
trans-Acting proteases	Primary virus (SINV) nonstructural protein (NSP2) rapidly degraded uncleaved P123 protein of superinfecting virus	224–226
Interference due to individual viral proteins		
BHV-1	Expression of BHV-1 glycoprotein D in MDCK cells interfered with replication of BHV-1, pseudorabies virus, and HSV-1	172
Poxviruses	Heterodimers formed by viral A56 and K2 proteins at the cell surface resisted superinfection	229, 230
WNV	Long-term incubation of superinfecting virus with primary virus-containing cells generated variant viruses that could overcome superinfection exclusion	231
Competition for cellular factors	DENV2 and DENV4 coinfection of mosquito cells resulted in reduced replication of both viral strains	142
Nonspecific dsRNA	Administration of both sequence-specific and non-sequence-specific dsRNA in bees resulted in lower viral titers; treatment with nonspecific dsRNA in adult bees resulted in enhanced survival following deformed wing virus infection	227, 228
RNAi		
DENV	DENV NS4B protein exerted a suppressive effect on RNAi response	232
FHV	FHV B2 protein prevented Dicer-2-mediated cleavage of long dsRNA as well as loading of siRNA into RISC	233, 234
Dicistro viruses	Encode protein 1A, which interacts with Dicer-2 or AGO2	235
Multiple flavivirus infection in insects	Generate cDNAs from the defective genome that are eventually transcribed by host transcription machinery to produce small dsRNAs, the source that induces the Dicer-2/RISC apparatus (RNAi pathway) that eventually regulates virus replication	236, 237
Interference by temp-sensitive mutants	Viral mutants that acquire dominance over wild-type virus	238–245

wild-type strain as well as that of heterologous wild-type strains. The interference occurred intracellularly without any role of DI particles or interferons and was directed exclusively against FMDV (139). Intrinsic interference may occur between similar, closely related, or unrelated viruses (140–144) (Table 2).

In non-interferon-mediated viral interference, competition between two viruses exists for the metabolites, replication sites (205), or those host factors that support virus replication (148, 167, 206–217). One virus modulates the host machinery in its favor, thereby interfering with the replication of other coinfecting viruses. A requirement for common cellular factors for unrelated viruses indicates that heterologous viral interference can also occur (1). Besides competition for cellular factors, several other mediators of viral interference are also known. These include DI particles (218), RNA interference (RNAi) (219–223), *trans*-acting viral proteins (224–226), and nonspecific dsRNA (227, 228), and these are listed in Table 3.

Viral interference usually occurs at specific steps of the virus replication cycle. These include attachment (144, 246–259), entry (217, 260–263), genome replication (167, 217, 231, 264–268), and budding (269). However, the infection may also invoke inhibition of multiple steps. For example, infection with recombinant Semliki Forest virus (SFV) inhibited attachment, entry, and uncoating in the subsequent secondary infection (217).

Superinfection exclusion. Superinfection exclusion is a phenomenon by which an established viral infection interferes with a second, closely related virus infection (159). This phenomenon occurs in both plant and animal viruses (270–272) and has important consequences for virus replication, pathogenesis, and evolution. It affects genome diversification, antiviral drug resistance, and evasion of vaccine-mediated immune responses. The members of a particular virus family may differ in their ability to exclude a superinfecting virus (217, 231, 267, 273–276) (Table 4). For example, infection with the

TABLE 4 Superinfection exclusion

Virus	Mechanism of exclusion	Reference(s)
Bovine viral diarrhea virus	Primary virus blocked entry and RNA synthesis of the superinfecting virus	283
Hepatitis C virus	Presence of primary virus RNA/proteins resisted superinfection	266, 275
Rubella virus	Exclusion occurred after entry but before accumulation of detectable amt of viral RNA	267
Semliki Forest virus	Superinfection exclusion occurs at the level of binding and endosomal fusion	217
Sindbis virus	Superinfection exclusion is mediated via viral nonstructural proteins (proteases)	167, 284
Measles virus	Superinfection exclusion is mediated via downregulation of CD46 (cellular receptor)	255, 285
Borna disease virus	Selective inhibition of polymerase activity of incoming viruses	264
HIV-1	HIV-1 Nef interferes with the superinfecting virus at the level of viral entry by downregulating CCR5, the major HIV-1 coreceptors	248, 254
Vaccinia virus	Superinfecting virus is unable to carry out its DNA synthesis and early gene transcription	271
West Nile virus	Competition for the cellular factors that are required for synthesis of the viral genome	231
Junin virus	No superinfection exclusion observed (virus failed to downregulate transferring receptor and thus was unable to resist superinfection)	286
	Persistently infected K3 cells excluded homologous arenavirus and antigenically related Tacaribe virus with an unknown mechanism	287
	Primary virus blocked protein synthesis by the superinfecting virus	277
Classical swine fever virus	Dysregulation of innate immune response with an unknown mechanism	279
Influenza A virus	Expression of neuraminidase by primary virus blocked secondary virus attachment to the host cells	288
Vaccinia virus	Primary virus infection blocked fusion of the viral (secondary virus) and cellular membranes	229
	Expression of A33 and A36 proteins in infected cells pushes the superinfecting virus particles on actin tails toward neighboring cells.	289
	Primary virus gene products inhibit early gene expression by the superinfecting virus	271, 290–292
	Heterodimer formed by the primary viral proteins (A56 and K2) at the cell surface inhibited secondary virus entry	293–296
Alphaherpesviruses (HSV, PRV, and EHV)	Glycoprotein D-mediated receptor interference	172, 297–299
Alphaherpesviruses (HSV and PRV)	Expression of immediate early viral genes (ICP0, ICP4, ICP22, and ICP27) resists superinfection with unknown mechanisms	300
PIV3 and NDV	Primary virus hemagglutinin-neuraminidase protein inhibited attachment of the superinfecting virus	301, 302
Alphabaculoviruses	Primary virus infection inhibited budding of the superinfecting virus	269
Citrus tristeza virus	Viral protein p33 mediates superinfection exclusion with an unknown mechanism	272, 273
Deformed wing virus (lethal and nonlethal types)	Lethal infection results in death of honey bees; prior infection with nonlethal DNV resists superinfection by a lethal deformed wing virus	303

Old World arenavirus results in downregulation of its receptors (α-dystroglycan) and thus induces resistance to superinfection. To distinguish coinfecting viruses at the level of transcription and translation, Gaudin and Kirchausen (286) developed a dual-reporter assay. They observed that, in contrast to infection with the Old World arenaviruses, infection with New World arenavirus (Junin virus [JUNV]) in A549 and Vero cells did not downregulate transferring receptors, and thus the cells were unable to resist superinfection. In contrast, persistently infected (with JUNV) cells did exclude superinfecting homologous or antigenically related arenavirus (277). Likewise, Env-, Vpu-, and Nef-mediated downregulation of the CD4 receptor resulted in HIV-1 superinfection exclusion (278). *In vivo* evidence of superinfection exclusion is rare (279–281). Examples include pigs persistently infected with classical swine fever virus (CSFV), which exclude vaccine strains upon immunization (282). Moreover, persistently infected pigs may also exclude highly virulent CSFV upon challenge infection (279). Viral and cellular factors that mediate superinfection exclusion in diverse groups of viruses are summarized in Table 4.

Superinfection suppression. The instance where persistently infected cells withstand challenge of a heterologous virus is termed superinfection suppression. Superinfection suppression has been observed between densovirus (DNV) and dengue virus (DENV). Persistently DNV-infected cells resist DENV challenge (with a reduced rate of DENV-2 infection, decreased DENV-2 production, and reduced mortality) in insect cells (147, 304). However, the superinfection suppression between DNV and DENV, as well as

that between infectious hypodermal and hematopoietic necrosis virus or (IHHNV) and white spot syndrome virus (WSSV) (176), should be referred to as superinfection disease suppression because the most prominent outcome is decreased disease severity rather than decreased viral infection (147).

Interference due to vaccination (live-attenuated viruses). The poliovirus vaccine strain is known to restrict the growth of standard (WT) virus (305). Later, following vaccination, this interference is achieved by stimulating antibody production that restricts the growth of the secondary virus. This evidence was derived primarily from field trials in which large-scale immunization campaigns against polio were found to displace antigenically unrelated enteroviruses (306). In addition, enteroviruses also interfered with poliovirus vaccines and led to vaccine failure (306). A similar phenomenon has been experienced with diverse groups of viruses, such as NDV (307), IAV (308), and DENV (268). However, the interference varies with the cell types and virus prototypes involved. Consequently, understanding viral interference is of utmost importance for the formulation and recommendation of any combination of vaccines (159).

Enhanced Virus Replication

Competitive inhibition is not the only outcome of coinfection (Fig. 1). Compared to single infection, CMV/HSV coinfection results in enhanced virus replication and virulence (309). Likewise, La Crosse virus (LACV) and Sindbis virus (SINV) coinfection in C6/36 cells resulted in enhanced SINV replication (166). In a study by Goto et al., human parainfluenza virus 2 (hPIV2) infection-associated cell fusion facilitated IAV replication and modulated pathological consequences (197). In another study, the simultaneous inoculation of culex flavivirus (CxFV) and West Nile virus (WNV) facilitated WNV transmission (168), although prior infection with CxFV had no effect on WNV replication.

Persistence

Contrary to the case in acute lethal infections, where virus particles are eventually cleared either by the immune system or by elimination of the host, persistently infected cells harbor virus for long times without clearance (1), thereby facilitating viral transmission to new hosts (236). Viruses isolated from persistent infections usually impede growth of the standard virus (242, 310–317). Since these viruses have managed to outgrow wild-type virus, they dominate over the parental virus in acute infections (245). Due to the inability to shut off the host cell machinery, persistent viruses have a reduced ability to kill infected cells.

DNV persistently infects mosquito populations, and this serves as a good model to study susceptibility to other viral coinfections in persistently DNV-infected cells (145). Compared to naive cells, persistently DNV-infected cells resist CPE formation upon DENV challenge (146, 147). The molecular mechanism underlying viral persistence is not completely understood. One potential mechanism is the activity of DI particles (318). Studies on flock house virus (FHV) suggest involvement of both host and viral factors in the maintenance of viral persistence (319–321). During establishment of *in vitro* persistent infection, the FHV genome remains unaltered; the mutations in the viral genome start accumulating after several successive passages (319), suggesting that a modified cellular environment, rather than virus itself, is crucial in establishing persistent infection. Following infection, ongoing virus replication is blocked either by the elimination of infected cell or by a host-directed RNAi response. Studies by Goic et al. suggest that FHV persistence in *Drosophila melanogaster* cells is accomplished through combined use of the RNAi and reverse transcriptase activity (237). Diverse RNA segments of FHV genome are reverse transcribed by long terminal repeat (LTR) retrotransposons. The resulting DNA molecules integrate with the host genome (322). Alternatively, the viral genome may be maintained as extrachromosomal circular DNA molecules (323). In both the cases, the viral DNA is steadily transcribed and produces dsRNA. These

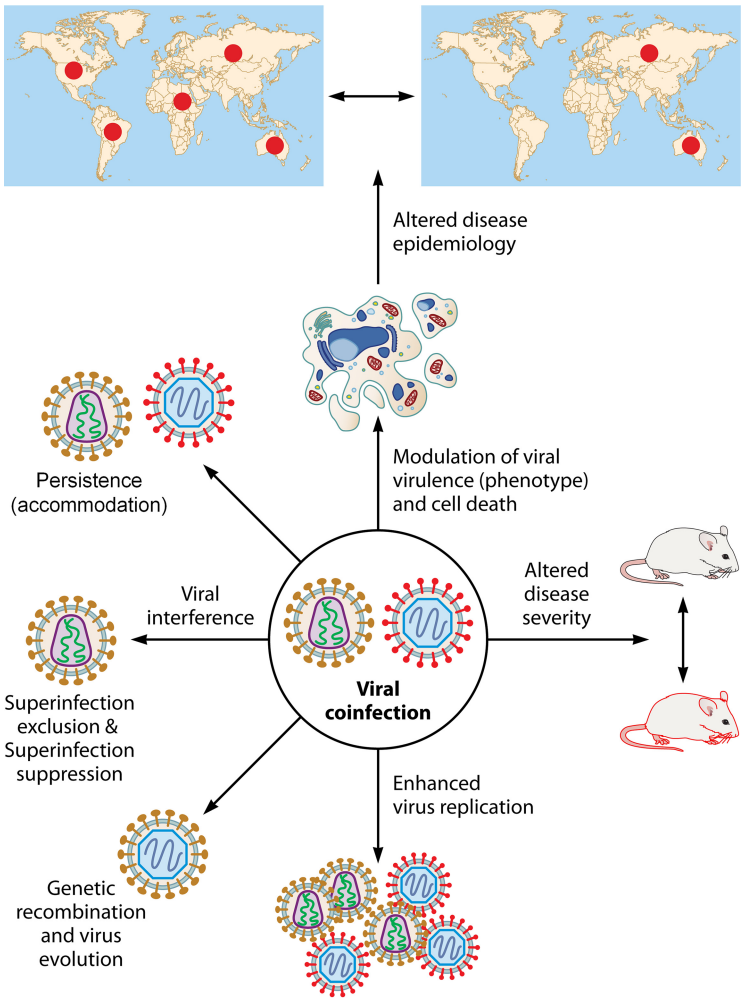

FIG 1 Virological outcomes of coinfections. Coinfections involving viruses may have several virological consequences. The most common outcome of coinfection is viral interference, where one virus competitively suppresses replication of the other confecting viruses. Interference between closely related viruses eventually results in elimination of the secondary coinfecting virus and is referred to as superinfection exclusion. The instances where persistently infected cells withstand the challenge of a heterologous virus are termed superinfection suppression. Besides diminished viral replication (interference), coinfections with certain viruses may also trigger enhancement of the replication of one or both of the confecting viruses. In several other cases, coinfection has no effect on the virus replication, and thus all the coinfecting viruses can coexist (accommodation). Coinfection may modulate viral virulence and cell death, thereby altering disease severity and epidemiology. However, genetic recombination between coinfecting viruses depends on the similarity between the coinfecting viruses.

dsRNA structures are eventually sensed by the RNAi machinery to block viral replication. Blocking of reverse transcription prevents the emergence of chimeric DNA, hence interrupting viral persistence (237).

Active, persistent infection by multiple viruses without any apparent signs of illness is referred to as viral accommodation and is commonly observed in arthropods (148) and shrimp (147, 304, 324, 325). There is little evidence that shrimp or other arthropods possess an immune system (326), but exposure of the shrimp to inactivated virions or envelope proteins can result in short-lived resistance to viral challenge (327). However, persistently infected shrimp only resist viral challenge until they remain infected (328), and there is no system equivalent to immune memory. In shrimp, mortality from viral diseases is an outcome of virus-triggered apoptosis (147, 329–331), and the viral accommodation that prevents triggering of apoptosis is not understood. The phenomenon of viral accommodation suggests that multiple viruses can stably coexist in the

same cell and that the possibility of genetic exchange between them depends on the degree of similarity between coinfecting viruses.

Modulation of Cell Death

In retroviruses, viral DNA integration in the host genome is catalyzed by both viral (integrase) and cellular (DNA-dependent protein kinase [DNA-PK]) factors. The initial events during viral DNA integration are sensed as a DNA damage response by the host, and this results in cell death (apoptosis). By promoting aggregation of the unintegrated viral DNA, superinfection exclusion in retroviruses may be employed to prevent cell death (332). In HIV-1, superinfection of primary T cells results in an increased level of apoptosis (274). One potential reason for HIV-1 inhibition of superinfection is to block premature cell death so that the virus may get sufficient time for replication.

Change in Virus Phenotype

Plaque assay is one of the most common methods to quantify virus particles. It is generally believed that a plaque represents a single infectious unit. As such, the number of plaques is believed to have a linear correlation with virus dilutions. However, a recent study has demonstrated that a plaque may contain multiple parental viruses. This possibly occurs due to the formation of virus aggregates, because even at an extremely low MOI, 5 to 7% of the poliovirus plaque population was found to be associated with multiple parental viruses (333). Coinfection with heterologous viruses (separate virus stock) may also result in altered plaque morphology, as seen with IAV and cowpox virus coinfection in BHK-21 cells (334). Likewise, plaques were small and opaque when persistently rubella virus-infected Vero cells were superinfected with another homologous virus (267).

Altered Disease Severity

In most instances, the contribution of coinfection at increasing or decreasing disease severity is difficult to determine. For example, in a PPRV/FMDV dual infection in goats, we noticed ~50% fatality (14). The fatality rate in PPR-affected sheep or goat flocks varies between 10 and 90% (335). Except in some young animals, FMDV usually does not cause any fatality in sheep and goats (336), so any role of FMDV/PPRV coinfection in fatality in goats could not be determined (14). Several other reports also suggest unaltered disease severity in mixed infections (337–342). Conversely, compared to the case for monoinfection, a higher rate of hospitalization/admission to the intensive care unit has been reported following multiple infections in humans, for example, coinfections with TTV, norovirus, and adenovirus (343), RSV and human metapneumovirus (hMPV) (174), IAV (344), and multiple respiratory viral agents (345). HIV-1-infected individuals, especially those with diminished $CD4^+$ counts, also have a high risk of influenza virus (192) and HCV (346) infection. In contrast, a less severe clinical impact of viral coinfections has also been reported (341, 347).

Hepatitis B virus (HBV) and HCV coinfections are quite common due to their shared mode of transmission. Compared to monoinfection, HBV/HCV coinfection results in more severe fibrosis and cirrhosis as well excess liver-related mortality (348, 349). Moreover, previous HBV infection (based on antibody detection) has also been also shown to significantly enhance the risk of decompensated cirrhosis (204). Clinical examination of HBV/HCV-coinfected patients suggests reciprocal replicative suppression (interference) (350). However, in an *in vitro* model (Huh-7 cells) of coinfection, both HBV and HCV could propagate in the same cell without any interference (201). Therefore, it was concluded that viral interference observed clinically in HBV/HCV-coinfected patients is mediated via host immune responses.

Experimental studies on viral coinfections are rare. In one study, reovirus and SARS-CoV infection in guinea pigs resulted in rapid death of the animals (351). Another experimental viral coinfection was described for vaccinia viruses (VVs). Based on plaque size and virulence in mice, two distinct groups of vaccinia viruses (group I and group II) that are associated with the exanthematous outbreaks in cattle are known (352, 353).

Coinfection of these two vaccinia viruses was reported in a natural outbreak in horses (353, 354). A mouse model of infection demonstrated more severe disease in coinfected (with vaccinia virus subtypes) than in monoinfected mice (200).

Altered Disease Epidemiology

By influencing disease severity and transmissibility and vaccine effectiveness, mixed infections may impact disease epidemiology. For a competition to succeed among multiple viral strains, they must be prevalent in the same geographical region, infect corresponding hosts, and target the same cells within that host. Viruses such as the DENV, with multiple variants and circulation across wide geographic regions, meet all these criteria (355–358). In nature, both humans and vectors (insects) are infected by multiple DENV subtypes (359–362). One major discrepancy between humans and vectors is that in the former, virus is cleared by the immune response, whereas in insects, it may persist for a long time. Therefore, vectors serve as a mixing vessel for any competition to take place between diverse viral strains. Since DENV2 and DENV4 coinfection results in competitive suppression, colonization of new viral strains may be blocked in those areas where mosquitoes are infected with multiple endemic DENV strains (142, 363–365).

Natural coinfection of rhinovirus and influenza virus does occur frequently in humans, but the situation is transitory (366), because rhinovirus negatively affects influenza virus replication (366). However, depending on the nature of the virus prototypes involved, coinfected hosts may shed more transmissible molecules than the singly infected host, and this can result in a higher disease prevalence (367). To comprehensively understand the significance of viral coinfections in epidemiology, further studies in natural populations are needed.

Genetic Recombination and Virus Evolution

Coinfection of a single cell with multiple viral strains allows genetic recombination, a major event driving viral diversity and escape from available antiviral drugs and vaccine-induced immunity (368–370). With segmented viruses, reassortment of the viral segments is a major source for the generation of novel viruses (371–373). The major influenza A pandemics in 1957, 1968, and 2009 all emerged from reassortment of viral segments (374). In influenza virus-infected cells, the efficiency with which a given neuraminidase (NA) removes sialic acid receptors determines reassortment between two or more viruses. However, the addition of an NA inhibitor in virus-infected cells can reduce viral titers and enhance superinfection and hence reassortment events (288).

Novel strains of poliovirus have been identified during early periods of excretion, and these appear to be generated due to recombination between poliovirus types 2 and 3 (375). Similarly, recombination between a persistently infected bovine viral diarrhea virus (BVDV) (noncytopathic form) and a vaccine strain resulted in the formation of a cytopathic form of BVDV. This led to lethal mucosal disease (376). Superinfection exclusion thus prevents the generation of viral diversity and detrimental recombination events, as well as maintaining cellular resources for primary virus infection. However, viruses such as HIV-1 generally replicate in short-lived T cells, and resistance to superinfection, which occurs primarily due to downregulation of CD4 receptors, barely reduces the recombination frequency (274).

Importin-α7, a cellular factor, is critically required for efficient IAV replication. Upon IAV challenge, importin-α7-knockout mice developed more severe disease than wild-type mice. In addition, virus recovered from the challenged mice was more virulent. This might have occurred due to more frequent recombination events and increased probability of superinfection in knockout mice (377). This evidence suggests that host-directed antiviral therapy may also result in the generation of more virulent viral phenotypes and hence should be considered carefully.

The impact of interstrain competition must be quantified in the epidemiological settings, as these may eventually influence long-term virus persistence and emergence

of virus variants. It is worth studying these effects *in vivo* in connection with the host immune system.

Factors Influencing Outcome of Coinfections

Virus dose and the time lag between coinfecting viruses. The time gap between coinfecting viruses is a major factor which influences viral interference. When wild-type and mutant SFV (SFV-tr) strains were added together at an MOI of 5, all the cells became infected, but if wild-type SFV was added 15 min after SFV-tr, fewer than 30% of cells were infected. Consequently, 15 min was enough to establish interference in most cell types (217). Similarly, instead of coinfection, infection with FMDV at 12 h after PPRV infection induced viral interference (14). In vaccinia virus superinfection, the secondary virus could not replicate at all if it was applied 4 h later (271).

The efficiency of viral interference also depends on the virus dose. Thus, when secondary virus was infected at a 10-fold larger amount (MOI = 50), the resistance to superinfection was overcome in a majority of the cells even when the secondary virus was applied 30 min later (217). At equal multiplicities of initial infection, DENV-3 and chikungunya virus (CHIKV) coinfection resulted in copersistence, with a similar result at higher CHIKV and lower DENV-3 infection levels. However, at lower CHIKV and higher DENV-3 infection levels, DENV suppressed CHIKV replication (143).

Cell types. A major factor which influences viral interference is the cell type used for coinfection. For example, Vero and BHK 21 are permissive cell lines for PPRV and FMDV, respectively. During PPRV and FMDV coinfection, a reciprocal competitive suppression (interference) occurs in BHK21 and Vero cells, respectively (14). In HIV-1 superinfection, Vpu and Env were found to more significantly affect downmodulation of the $CD4^+$ in peripheral blood mononuclear cells (PBMCs) than in the Jurkat T cells (274). Likewise, $CD4^+$ downmodulation by HIV-1 is more profound in Jurkat T cells than in PBMCs (378), suggesting a role of cell type in viral interference (379). Sperm proteins, human T-lymphotrophic virus (HTLV), Epstein-Barr virus (EBV), and CMV share similarity with the HIV-1 cellular receptor $CD4^+$ present on T helper lymphocytes (380). The binding of HIV-1 to these additional $CD4^+$ homologues on sperm or other coinfecting viruses allows it to infect additional cell types which are not infected normally (380).

Route of infection. The route of infection also impacts the consequences of viral coinfections. For example, LACV-infected mosquitoes (*Aedestriseriatus*) remained sensitive to secondary heterologous infection with bunyaviruses if the primary virus was administered transovarially (381). However, when inoculation of the primary virus was by the intrathoracic route, the mosquitoes resisted superinfection (382). In CxFV/WNV coinfection, when the mosquitoes were inoculated by the intrathoracic route, both CxFV and WNV were present in the saliva (168). However, CxFV was not detectable in the saliva of singly infected mosquitoes, suggesting that CxFV infects the salivary glands by "piggybacking" on WNV (168).

Age. In humans, coinfections are more commonly observed in children than in adults (11). A study carried out in a population ranging from 0 to 105 years suggested that children <5 years showed an increased rate of viral coinfection than older persons (345). In another study, it was observed that the propensity for viral coinfection was greater in children age 6 to 24 months than in infants (0 to 6 months) (347). A study by Zhang et al. (383) demonstrated that among children <3 years of age, the 13- to 24-month age group had relatively higher rates of viral coinfections than the 8-to 12-month or 25- to 36-month age group.

Rate of virus replication and CPE formation. Cytolytic viruses rapidly deplete cellular resources and induce cell death. If coinfecting viruses significantly vary in their replication cycle length, the one with the shorter life cycle will persist because other coinfecting viruses with longer cycles will be prematurely terminated. For example, in PPRV/NDV coinfection, the relatively long replication cycle of PPRV ($\sim$24 h, compared to 8 h for NDV) led to its removal upon long-term cell culture passage in Vero cells (14).

In buffalopox virus (BPXV) infection, evidence of both peak virus titers and CPE formation is observed at $\sim$48 h postinfection (hpi) (14). In PPRV infection, although

evidence of the new progeny virus particle is observed quite early, at ~24 hpi (but with very low titers), virus titers progressively increase until 7 days postinfection (dpi), and the CPE cannot be observed until 4 to 5 dpi. Therefore, in BPXV/PPRV coinfection, faster CPE formation by BPXV eliminated PPRV on long-term passage (14) in Vero cells.

IMMUNOLOGY OF VIRAL COINFECTIONS

Animals and humans are exposed to a variety of environmental pathogens and thus have a different infection history than mice grown in a containment (specific-pathogen-free) facility. Upon encountering an antigen following viral infection, naive T cells become activated effector cells, ultimately leading to memory T-cell formation. Memory responses elicited as a result of previously encountered pathogens play a significant role in deciding the type and magnitude of immune responses mounted against the subsequent infections (384). Immune responses to previously experienced pathogens can modify responses made against unrelated pathogens. This is referred to as heterologous immunity, and it can occur between related or unrelated viruses or between viruses or other types of pathogens (2).

The heterologous immune response may provide protective immunity or may lead to immunopathology, depending upon multiple factors. These include the type of viral infection (385), dose of virus (386), stage of infection (387), and, in some circumstances, age of the host (388). Diverse arrays of mechanisms are involved, and almost entire immune cell types are known to participate and modify the outcome of infections. Altered immunodominance hierarchies, a remodeled T-cell receptor (TCR) repertoire, and cross-reactivity are the major changes recorded during heterologous infections. In this section, we discuss current knowledge and recent developments in heterologous immunity related to concurrent as well as sequential infections. Several mechanisms and immune outcomes with specific examples are mentioned, and these are related to virus control measures, prevention of inflammatory consequences, and implications for diagnostic and vaccination strategies.

Immunity or Immunopathology

Certain heterologous viral infections result in protective immunity by employing mechanisms that include innate immune activation (389), bystander protection by activated $CD4^+$ or $CD8^+$ T cells (388), and cross-reactive $CD8^+$ T cells (390). However, in some instances, heterologous infections result in an unaltered immune response, suggesting that the viral coinfection had no significant consequences. Indeed, work with heterologous viral infections such as IAV, murine cytomegalovirus (MCMV), lymphocytic choriomeningitis virus (LCMV), and vaccinia virus (VV) has convincingly demonstrated that IAV-immune mice were protected against VV but not MCMV. In fact, IAV suppressed the clearance of MCMV and LCMV. In addition, the cross talk was not reciprocal, with virus A affecting responses to virus B but not the reverse effect. For example, LCMV-immune mice may resist VV challenge, but VV-immune mice remained fully susceptible to LCMV challenge (391).

Immunopathology is yet another facet of heterologous immunity where viral coinfections culminate in severe and prolonged lesions. Following viral infection, an effector $CD8^+$ T-cell response is generated, which helps in antigen clearance. Upon clearance of the antigen, regulatory responses are induced, which suppress effector responses, thereby preventing collateral damage due to the excessive cytokine production by the activated effector T cells. Thus, the infection is resolved with minimum tissue damage. However, heterologous infections under some circumstances could bring about an uncontrolled immune response and consequent development of immunopathology. This happens with DENV infection and in flu infections both of which involve development of a cytokine storm (392). When infected with MCMV, LCMV-immune mice may exhibit enhanced immunopathology and augmented viral loads, and MCMV-specific memory T-cell inflation was suppressed (390). Similarly, LCMV-immune VV infected mice experienced a more severe outcome (393). The memory T-cell response in IAV-immune LCMV-infected mice enhanced lung immunopathology.

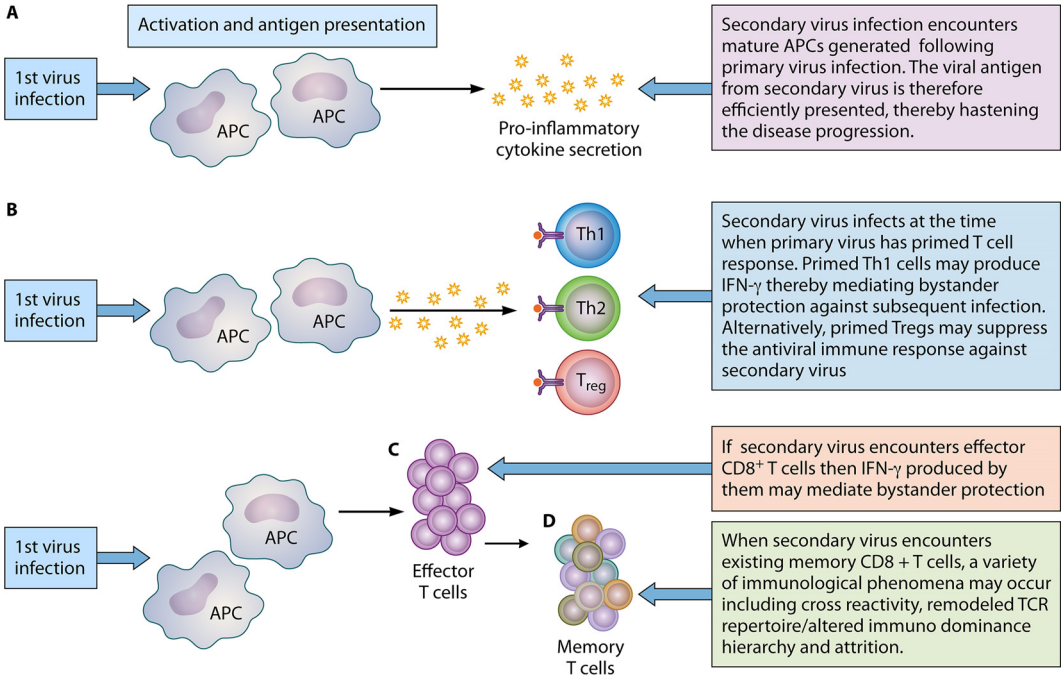

FIG 2 Immunological outcomes of heterologous viral infections. The immunological outcome is dependent upon the stage at which a subsequent viral pathogen is encountered. (A) Primary virus infection activates APCs. Subsequent infection entering following maturation of APCs culminates in efficient antigen presentation that may hasten the disease progression (immunopathology or protective immune response, depending on the nature of the immune response mounted). (B) Upon encountering antigen, activated APCs secrete cytokines that ultimately influence the type of T-cell differentiation. When the new incoming pathogen encounters already polarized T helper cells, bystander protection is mediated by these polarized Th1 cells. However, encounter with a polarized regulatory T cell can suppress immune responses against new pathogen. (C) Bystander protection from IFN-γ production may also be mediated under conditions where subsequent heterologous infection occurs during an ongoing effector $CD8^+$ T-cell response. (D) When a new virus infects the host with an established memory $CD8^+$ T-cell pool as a result of prior viral infection, the outcome may be cross-reactivity (can be protective or pathological), a remodeled TCR repertoire, or an altered immunodominance hierarchy. Inversely, the incoming pathogen can result in attrition (type 1 IFN dependent) of preexisting memory $CD8^+$ T cells.

In humans, cross-reactivity in EBV and IAV epitopes (394) resulted in the amplification of the cross-reactive T cells that had subsided affinity for the cells expressing viral antigen and thus were inefficient in clearing infection. Primary DENV infection generates a high-avidity $CD8^+$ T-cell response. Upon a secondary DENV infection, the augmentation of the primary virus-specific $CD8^+$ T cells occurs due to cross-reactivity, rather than the cells specific for secondary infection.

Whatever the mechanism, the particular sequence of infections, time interval between infecting viruses, and route of infection are the decisive factors that determine the outcome (pathological/protective) of heterologous viral infections. The dose of virus may also affect the extent of immunopathology. In a study with LCMV clone 13 infections in a mouse model, a low dose of the virus generated strong effector T-cell responses that efficiently cleared the virus. High viral doses, on the other hand, resulted in T-cell clonal exhaustion, viral persistence, and limited immunopathology. Interestingly, intermediate viral doses could elicit an immune response with a lower rate of exhaustion and provided sufficient time for profound collateral damage to occur in the lung and liver, often resulting in death (386).

Net Outcome of Viral Coinfections

The net outcome depends upon the stage at which the subsequent pathogen is encountered (Fig. 2). For instance, the second incoming pathogen may enter at a stage when prior infection has primed the innate immune responses or at a stage when it encounters a polarized helper T-cell subset. Preexisting primary virus-specific $CD4^+$ and $CD8^+$ T cells are also known in some cases to provide bystander protection against the subsequent pathogen, although the mechanisms involved are not understood (395).

Primed innate immune responses. Innate immunity plays a crucial role in safeguarding against viral infections. Following pathogen recognition by specific receptors, various inflammatory cascades are triggered that eventually results in secretion of cytokines and chemokines, activation of antigen-presenting cells (APCs), and recruitment of innate immune cells. These can, in turn, modulate responses to subsequent viral infection. The extent of this modulation depends largely upon the time gap between the two infecting viruses. Simultaneous coinfection, for instance, may lead to higher viral loads and increased immunopathology. In an alternate situation, primary infection may lead to maturation of the APCs that eventually augment antigen presentation upon subsequent viral infection. For example, in one study, LCMV infection caused stimulation of Kupffer cells, recruitment of T cells and NK cells, and enhanced production of tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), and IFN-α/β. This scenario favored faster elimination of HBV in the coinfected animals (396). Similarly, herpesviruses are known to establish persistent infections and latency. Subsequent secondary infection of the persistently infected host may culminate in an altered immune response. For example, herpesvirus latency is known to enhance the basal activation status of innate immune compartments and thus protective immunity to subsequent secondary infections (389). However, this concept is not generally accepted.

Polarized T helper subset. $CD4^+$ T cells exert innumerable activities in antiviral immunity (397). These include mediating either a protective or immunopathological role in a setting of heterologous infection. Prior infection of the host may lead to APC activation. These activated APCs undergo cytokine secretion that eventually drives TH subset differentiation, i.e., TH1, TH2, TH17, or regulatory T cells (Treg), depending upon the cytokine milieu contributed by the innate immune cells that respond to the primary viral infection. A preexisting polarized T helper immune response generated against primary virus infection may induce bystander protective immunity (387) or an immunopathological response upon exposure to a subsequent secondary viral infection. Adoptive transfer of $CD8^+$ and $CD4^+$ T-cell subsets from LCMV-immune to naive mice revealed heterologous immunity to Pichinde virus (PICV) and VV in the recipients (398).

An exacerbated immune response to viral infections is controlled by several regulatory mechanisms, which otherwise might result in immunopathology and autoimmune disease. These regulatory mechanisms include the expansion of regulatory T cells (399, 400) and various inhibitory protein-mediated interactions. For example, the Tim3/galectin 9 (401–405), PD1-PDL1 axis (406), and CTLA-4 and CD80/86 (406) interactions strongly influence the outcome.

Regulatory T cells. $FoxP3^+$ $CD4^+$ T cells (407) play a pivotal role in influencing the amplitude of T-cell responses to viral infections (2). Treg expand during viral infection, with an enhanced suppressive function (400). Such Treg can alter the magnitude and other features of effector T-cell responses and limit immunopathology upon exposure to subsequent heterologous infection. This Treg-mediated impact either may positively influence the outcome or can be detrimental to the host (387). However, the sequel of coinfection may be influenced by numerous other components such as the infection status, dosage of the pathogen, genetic makeup, and immunological condition of the host in addition to the presence of other concurrent infections (408).

Treg that have expanded during primary infection can suppress bystander responses (408) induced by exposure to a subsequent heterologous pathogen. Treg depletion at both the acute and memory stages of an antiviral immune response may lead to enhanced $CD8^+$ T-cell responses (400). Viral infection can thus temporarily dampen immunity to subsequent viral infections. Upon IAV infection, the expanded Treg affect the nature and magnitude of the effector responses, as well as their contribution to lung immunopathology following heterologous infection. In line with this, Treg induced following IAV infection mitigate the T-cell responses following heterosubtypic IAV challenge and thus diminish pathology following heterologous IAV challenge (409).

Treg were also shown to regulate virus clearance and immunopathology in persis-

tent viral infections (410, 411). Accordingly, with a heterologous infection model of persistent and nonpersistent viral infections, Treg generated as a result of past infections diminished subsequent immune responses and lung immunopathology upon exposure to heterologous virus infection (412). With an IAV and LCMV heterologous infection model, when Treg were depleted in IAV-immune mice and subsequently infected with LCMV, unexpectedly, lung pathology was reduced. The LCMV-specific $CD8^+$ T-cell responses in the spleen were significantly reduced but not those in the mesenteric lymph nodes (mLNs). The explanation advocated was inefficient effector T-cell trafficking to lymph nodes due to the absence of Treg in both the naive mice and LCMV-infected IAV-immune mice. The study thus confirmed the established role of Treg in regulating effector T-cell exit from lymph nodes (413). Moreover, there was no enhancement of virus-specific effector responses when IAV-expanded Treg were depleted during LCMV infection (414, 415).

The observations above contrast with the report where PC61 (anti-CD25) treatment inhibited regulatory T cells expanded by IAV infection, which resulted in extensive lung pathology upon subsequent LCMV challenge (412). The drastic decrease in the degree of lung pathology upon Treg depletion was attributed to the fact that the LCMV-specific $CD8^+$ T cells were overactivated and subsequently partially exhausted in mice immune to IAV and infected with LCMV.

In influenza virus-immune mice, infection with LCMV resulted in increased viral titers and lung pathology along with a modified cytokine profile in comparison to those in naive mice infected with LCMV. This was explained by the increased numbers of $CD4^+$ $Foxp3^+$ regulatory T cells in the lungs of influenza virus-immune mice compared to those in naive or LCMV-immune mice. Therefore, it is plausible that modulating the normal proportions of Treg and effector T-cell responses might have played a role in altering the responses in influenza virus-immune mice infected with LCMV. In this heterologous IAV/LCMV infection model, acute LCMV infection provided peak $CD4^+$ cells, $CD8^+$ cells, and Treg at day 3, which started decreasing at day 7, in the mLNs. However, in influenza virus-immune mice, the Treg persisted at elevated levels until day 9 following LCMV infection. Thus, in influenza virus-immune mice, heterologous LCMV infection resulted in altered Treg kinetics. This led to higher viral loads, increased proinflammatory cytokine and chemokine levels in the lungs, and ultimately immunopathology (409).

Preexisting $CD8^+$ T cells that mediate bystander protection. $CD8^+$ T cells are known to confer protective heterologous immunity. In an adoptive transfer model, transfer of $CD8^+$ and $CD4^+$ T cells from LCMV-immune mice to naive mice provided protective heterologous immunity to PICV in the recipients (398). Furthermore, in a mouse model of coinfections, CMV infection conferred protection against IAV infection. In the same study, CMV-seropositive human adults displayed increased antibody responses to influenza vaccination compared to those in seronegative individuals. The enhanced responses included $CD8^+$ T-cell activity and higher levels of IFN-γ. Thus, prior CMV infection has a beneficial effect on the immune system of young healthy humans. The mechanism of this CMV-mediated beneficial effect was attributed to the bystander protection offered by the IFN-γ (produced from CMV-specific $CD8^+$ T cells) (388), and in a parallel mice study from the same group of researchers, it was demonstrated that this $CD8^+$ T-cell-mediated protective immunity was completely abrogated in IFN-γ knockout mice. Alternatively, persistent infections may result in a modified inflammatory environment that leads to a change in dominance patterns. In a murine model of latent CMV infection, diminished $CD8^+$ T-cell responses were observed upon secondary heterologous infection by WNV, IAV, or human herpesvirus 1 (416).

Superinfection of persistently infected host with acute heterologous infection such as with IAV results in persistent activation and proliferation of virus-specific cells (388). Similarly, simultaneous infection of gammaherpesvirus and influenza viruses resulted in enhanced numbers of lymphocytes in peripheral blood, as well as $CD8^+$ $CD4^-$ T cells and $CD19^+$ $CD45^+$ B cells, in lungs of coinfected animals compared to singly infected

mice. This in turn generated higher levels of IFN-γ and antibodies and ultimately a stimulated immune system (417).

Attrition of preexisting CD8$^+$ T cells upon subsequent heterologous infection. It has been confirmed in several animal model systems that an incoming heterologous infection may deplete the preexisting CD8$^+$ T cells. This is referred to as attrition (418, 419). The finite space in the immune compartment supports the theory of attrition, where the subsequent viral infection induces interferon which in turn mediates apoptosis of memory T cells generated upon encountering primary virus infection. More than one class of interferons can also induce erosion of preexisting memory (420).

A cell surface receptor known as programmed cell death protein 1 (PD1) promotes apoptosis and serves as an immune checkpoint. It induces self-tolerance by inhibiting T-cell inflammatory activity. PD1 suppresses autoimmunity by depletion of autoreactive CD8$^+$ T cells in mice (421). In a mouse model of heterologous CMV/HBV infection, PD1 suppression was found to be upregulated by CMV-specific CD8$^+$ T cells, which was associated with enhanced apoptotic activity of these cells. Blockade of the PD1 pathway by anti-PDL1 antibody restored the proliferation and cytokine secretion by these CMV-specific CD8$^+$ T cells (422). Thus, besides type 1 IFN, PD1-mediated apoptosis is yet another mechanism proposed for attrition.

Attrition could have a catastrophic consequence for vaccine-induced memory. However, contrasting data with a prime-boost vaccination strategy with VV suggested that immunological memory can grow in size while still preserving memory for previously encountered pathogens (423). VV induces poor interferon responses, and moreover, it generates effector memory cells that are present outside the lymph nodes, in contrast to central memory located within the lymphoid compartment. The possibility of central memory being more tightly regulated and the rapid erosion of immunological memory require further investigation. Measles virus provides long-lasting protective immunity in humans (65 years). Likewise, memory B cells persist for an indefinite period after smallpox immunization, suggesting that memory responses do not need to erode rapidly. In conclusion, some researchers support attrition because the space in the lymphoid compartment is finite; however, others do not support attrition, and various theories are proposed to explain both facets, thus presenting the idea of controversy.

It was argued whether virus-specific CD4$^+$ memory T cells undergo attrition upon heterologous virus infection. However, data from mouse models indicate that LCMV-specific CD4$^+$ T cells are relatively stable following various heterologous virus infections and protein immunization. However, in contrast, under the same circumstances, LCMV-specific CD8$^+$ T cells were significantly reduced, suggesting that the T helper and cytotoxic memory cell pools are independently regulated (424).

Mathematical modeling studies indicate that upon each subsequent infection, viral clearance is challenged, and when the number of infections crosses a threshold, then viral control is completely abraded, thus supporting the loss of memory CD8$^+$ T cells upon each new incoming infection (2).

Common Phenomena Observed in the Setting of Heterologous Infection

Altered immunodominance hierarchies. Adaptive immunity is defined by the fact that a unique T-cell repertoire is established to a wide range of immunodominant epitopes. Immunodominance hierarchy depends upon the dose of antigen and numbers of T and B cells (425). Following a viral infection, multiple major histocompatibility complex class I (MHC-I) molecules are coexpressed along with the generation of numerous immunogenic peptides. However, the majority of the antiviral cytotoxic T lymphocyte subsets target only a few peptide/MHC class I complexes. This phenomenon where only limited peptide/MHC class I molecules are targeted by antiviral cytotoxic T lymphocytes is known as immunodominance. Among these peptide/MHC complexes, some epitopes are dominant and others are subdominant. For example, following IAV infection, although the primary CD8$^+$ T-cell responses to D^bNP_{366} and D^bPA_{224} epitopes are of equivalent size, after secondary challenge, the D^bNP_{366}-specific

T cells become the predominant responders. Naive hosts respond quite differently to antigen exposure than an antigen (heterologous)-experienced host. The presence of antigenically experienced cross-reactive $CD8^+$ T cells which compete with the proliferation of naive $CD8^+$ T cells partly contributes to altered epitope-specific hierarchies.

Remodeled TCR repertoire. The response to an antigen can be represented as the number of T cells that are recruited and the structure of their antigen receptors. Quantifying an immune response at the repertoire level is now becoming very common. The array of individual clonotypes with TCRs specific for a distinct peptide MHC epitope is known as the TCR repertoire (426). The repertoire varies considerably in constituent TCR frequency and diversity. A diverse TCR repertoire benefits the host in combating a large number of pathogens. Tools such as TCRdist have recently been developed, which could be used to calculate the similarity and differences of key features of T-cell receptors and to identify those T-cell receptors that could recognize similar epitopes (427).

Heterologous infection can lead to the broadening of an otherwise narrow repertoire by recruiting the nondominant clones but at the same time could narrow the repertoire due to cross-reactivity. Primary infection-associated repertoires can be remarkably altered by a new, heterologous infection. For example, the $NP_{205–212}$ epitope is encoded by both LCMV and PICV. This NP epitope elicits a TCR repertoire that is different in both infection types (LCMV and PICV) but is highly cross-reactive. Heterologous infection, i.e., infection of PICV-immune and LCMV-immune mice with LCMV and PICV, respectively, resulted in a narrow oligoclonal repertoire with clones having unpredictable TCR sequences. In this heterologous infection study, non-cross-reactive epitope-containing TCR repertoires were unaffected. However, cross-reactive $CD8^+$ T cells proliferated after heterologous challenge. On the other hand, minimal alteration in the repertoire was observed in mice following homologous viral challenge, and the expected TCR motifs were observed (428). In another study, PICV infection followed by heterologous LCMV infection resulted in dominance of a subdominant NP epitope (429). Thus, discrepancies may result from challenging or vaccinating hosts with distinct immunological histories.

Alteration in repertoire diversity can also follow homologous challenge. In a study with bluetongue virus (BTV), virus-specific $CD8^+$, but not $CD4^+$, T cells expanded during the recall responses to BTV challenge. In addition, primary responses elicited a wider range of repertoire for MHC-I and MHC-II epitopes than the memory response, where a narrowed repertoire was induced in a dominant motif in VP7 (amino acid position 139 to 291) (430).

Cross-reactivity. One of the several mechanisms proposed for the altered immunodominance hierarchies in heterologous infections is cross-reactivity. Cross-reactivity is the capacity of the TCR to recognize multiple peptide/MHC complexes, and this can occur in several different modes. These include the same TCR recognizing multiple peptide/MHC complexes or by molecular mimicry in which the TCR can bind to unrelated peptide/MHC complexes in a variable manner or may itself change the conformation within the flexible CDR3 loops.

Cross-reactivity may be advantageous as well as disadvantageous for the host. Cross-reactive immune responses in viral coinfections can either inhibit or augment the growth of new incoming pathogen (431). On the beneficial side, cross-reactivity could protect under conditions where a large number of pathogenic antigens and a limited TCR repertoire mounted by the host occur. On the harmful side, cross-reactivity may involve narrowing the TCR repertoire and consequently viral escape (428).

Cross-reactive $CD8^+$ T-cell response were shown to occur during coinfection with multiple homologous strains, such as in IAV and DENV strains. It also occurs between completely unrelated viruses. In humans, the BMLF1280 antigen of EBV cross-reacts with IAV epitope M158.13, which is HLA-A*0201 restricted. This results in the induction of cross-reactive T cells with a reduced affinity for virus antigen-expressing cells and inefficient viral clearance (394). Upon secondary DENV infection in humans, the $CD8^+$ T cells generated have a higher avidity to previously encountered DENV epitopes, and

thus these cells expand compared to those T cells for the newer serotype expressed by the current infection (406). Another example includes HCV and IAV (394). HCV infection demonstrates a variety of symptoms varying from subclinical to clinical. The infection either is cleared from the host or may become persistent. One study reported that patients with acute HCV mount a T-cell response recognizing a diverse group of peptides; however, patients with chronic HCV mount a narrow T-cell response directed against cross-reactive influenza virus and HCV epitopes (432). Thus, cross-reactivity regulates disease severity in acute and chronically infected human patients. Cross-reactive memory cells elicited by past exposure to infection could influence immune response to other infectious agents, and this could impact the efficacy of vaccines (433).

ADE. Virus-specific antibodies are well known to clear virus infection; however, a subneutralizing amount of antibodies can also augment virus infection and therefore disease severity. In antibody-dependent enhancement of infection (ADE), subneutralizing, cross-reactive antiviral antibodies bind to virion particles and facilitate infection of cells expressing Fc-γ receptors (Fc-γRs), including macrophages, monocytes, and some dendritic cell subsets. ADE usually occurs in patients who have preexisting antiviral immunity and are subsequently exposed to a heterologous virus. Alternatively, ADE can occur due to the presence of maternal antibodies in infants (434).

In DENV, the cross-reactive antibodies are hypothesized to promote DENV infection and antigenemia, which eventually result in severe DENV syndrome characterized by fever, hypotension, vascular leakage, thrombocytopenia, hemoconcentration, and end-organ damage (435). Besides ADE, cross-reactive, dysfunctional T-cell responses may also contribute to enhancing disease severity (435, 436). In addition, modifications on subtypes of cross-reactive IgG can also regulate interactions with specific Fc-γRs to influence disease severity (437).

Due to amino acid relatedness (nearly 43%) and cross-reactive antibodies between DENV and Zika virus (ZIKV), there is speculation that preexisting cross-reactive T cells and DENV antibodies can facilitate ZIKV infection. Bardina et al. (438) immunized STAT2$^{-/-}$ immunodeficient mice by injecting DENV- or WNV-immune plasma and subsequently inoculated them with ZIKV. The cross-reactive antibodies against WNV and DENV facilitated ZIKV replication and lethality. A higher incidence of ZIKV infection with more severe clinical manifestations (congenital malformations) was noticed in areas with a prior flavivirus infection, which could be explained by ADE. This, however, has not been substantiated.

Implications of Heterologous Immunity

Diagnostics and therapeutics. Physicians and clinicians usually do not consider more than one viral etiological agent for diagnosis and therapy. Nevertheless, the association between the presence of coinfection and increased/decreased disease severity is still unclear. Indeed, an adequate diagnostic procedure investigating diverse groups of viral pathogens is important for appropriate therapy (439).

During an ongoing HCV infection, HIV coinfection hastens the development of hepatic fibrosis. Thus, therapy in coinfected individuals demands judicious implementation of the therapeutic regime (440). In another study, it was demonstrated that TNF is vital for VV control in naive mice, yet in LCMV-immune mice TNF is not essential for VV clearance (441). Thus, anti-TNF therapy could be safe in such cases. If an immunopathological outcome is expected in diagnosed coinfections, appropriate interventions (anticytokine therapy) can be employed to prevent severe immunopathology (398, 442, 443).

Transplantation. Information regarding the previous infection history is also of vital importance in transplant recipients because successive heterologous viral infections result in increased numbers of alloantigen-specific T cells. These cells require tolerization before the graft transplant. Indeed, studies have shown that alloreactive immune responses elicited following viral infection could hamper tolerance induction (444). Several inhibitory mechanisms and costimulatory blockades (445–447) have also been performed, either singly or in combinations to enhance the rate of allograft survival. These attempts to disrupt T-cell activation could compromise T-cell-mediated antiviral

immunity in a host with already ongoing persistent viral infection, such as infection with Epstein-Barr virus (EBV), HCV, and CMV, but also for a new incoming heterologous infection. Thus, the choice of costimulatory blockades could have critical consequences for transplant recipients. Therefore, a thorough knowledge of heterologous immunity helps guide successful engraftment and management of transplant recipients.

Vaccination. Under the majority of circumstances, the infection history in humans and particularly animal species remains largely unknown. Thus, predicting the outcome of vaccination in heterologous infections in experienced hosts is challenging.

The efficacy of vaccines might be reduced due to immune-dominant alterations of undesired T-cell responses (443, 448) as a consequence of cross-reactivity. Cross-reactive T cells play a crucial role in pathogenic and protective immunity to heterologous infection. Hence, careful identification of such cross-reactive memory T cells could aid in vaccine designs. Thus, vaccines lacking cross-reactive epitopes (391) could be supplemented to formulate effective vaccination strategies (449). As discussed above, heterologous challenge can lead to erosion of the memory $CD8^+$ T cells generated against the previously experienced antigen. This erosion could be explained by space restrictions within the immune compartment, which, if true, would have disastrous consequences for memory $CD8^+$ T cells elicited in response to vaccination. This could mean that new incoming heterologous infection would displace the memory $CD8^+$ T cells generated against the target pathogen by vaccination and vice versa, where vaccination could displace the preexisting memory $CD8^+$ T cells elicited upon exposure to a previous heterologous infection.

With LCMV and vesicular stomatitis virus (VSV) prime-boost vaccination strategies, the enhancement of the memory $CD8^+$ T-cell compartment was demonstrated to harbor newly developed clones of effector memory $CD8^+$ T cells (423). In addition, attrition focused on secondary lymphoid tissue and the central memory population, whereas the prime-boost vaccination strategy mainly induced effector memory $CD8^+$ T cells that resided within the nonlymphoid compartment (423). Moreover, attrition is induced by viruses that are strong interferon inducers. Thus, it is noteworthy that vaccines which generate a strong interferon response could end up in causing attrition of preexisting memory $CD8^+$ T cells. However, this notion requires more study.

The induction of cross-reactivity and attrition are major concerns in vaccination. For instance, the VV vaccine is known to induce potent immune responses. Thus, such vaccines reduce the risk of infections and are likely to have heterologous impacts on the immune system. Smallpox immunization is advocated to lower the risk of asthma and malignant melanoma (450), due to heterologous effects of the vaccine on the immune system. However, we no longer vaccinate against smallpox, since the disease has been eradicated (451).

Similarly, the measles vaccine has an additional advantage; besides providing protection against measles, it could provide protective immunity against other, unrelated infections. Nevertheless, this wide-ranging beneficial effect could be abolished if the measles vaccine was followed by an inactivated diphtheria-tetanus-pertussis DTP vaccine (452).

MATHEMATICAL MODELS OF VIRAL COINFECTIONS

In order to better understand disease dynamics as well as to devise better therapeutic regimens, mathematical models of viral coinfections have also been developed (453). Most of the mathematical models involve HIV/HCV coinfection. Vickerman et al. first proposed a mathematical model for HCV/HIV transmission and concluded that sharing of needles/syringes is likely to increase HIV/HCV incidence in injecting drug users and that HCV infection indicates the risk of HIV infection (454). In another model, it was suggested that health care workers must be given sterile equipment (water filters and cookers) to prevent HCV infection (455). HIV loads impact the severity of HCV infection (456); therefore, treatment with highly active antiretroviral therapy (HAART) is specifically recommended to reduce the number of carriers (457). Mathematical models also suggest that the treatment efficacy influences the natural progression of HCV in HCV/HIV coinfection (456), and HAART is associated with a reduction in the transmis-

sion of HIV (458, 459). Moreover, HCV progressively induces a negative effect on human health, irrespective of the HIV status (460). In 2015, Birger et al. (461) refined a preexisting model of HCV infection by integrating dynamics of HIV and HCV coinfection as well as components of the immune system that clear infection. It was concluded that the propensity for HCV infection is greater in immunocompromised HIV-1 patients.

Rong et al. (462) presented a mathematical model for drug-sensitive and drug-resistant HCV. The model concluded that viral mutations acquired during the course of drug therapy have no major impact on the dynamics of different viral strains. Although low levels of HCV variants may be generated, they are liable to be completely suppressed due to fitness disadvantages (462, 463).

Pinky and Dobrovolny (464) developed a mathematical model to study the dynamics of IAV, RSV, rhinovirus, hPIV, and human metapneumovirus (hMPV) coinfections. The model suggested that one virus dominates over the other simply by being the first to infect, without involvement of viral interference or immune response. Rhinovirus, the most rapidly replicating virus, interferes with replication of other coinfecting viruses, while PIV, the most slowly replicating virus, is interrupted in the presence of other viral agents (464).

By considering that infection is cleared before initiation of the cellular regeneration, most of the prevailing mathematical models (for respiratory viruses) do not consider regeneration of the cells within the respiratory tract. In order to determine the effect of cellular regeneration on coinfection dynamics, Pinky and Debrovolny (465) investigated four mathematical models that incorporate distinct mechanisms of cellular regeneration. The models suggested that chronic illness is possible only with one viral species. Coexistence of multiple viruses in chronic conditions is unlikely to occur if the regeneration model is considered (465).

CONCLUDING REMARKS

Understanding the drivers of multiple infections and virus-virus interactions is an emerging field in virology. Classically, the laboratory examination of clinical specimens is biased to associate it with a single identified pathogen. Frequently, additional agents that contribute to the disease outcome are undetected. Due to viral interference, one virus infection may alter consequences of the other coinfecting viruses. Coinfection by related viruses may lead to genetic recombination/reassortment. This produces antigenic variants that can escape vaccine-induced immunity as well as the efficacy of antiviral drug therapy. Nonpathogenic divergent viruses present in clinical specimens may influence detection of pathogenic viruses, particularly when cell cultures are the diagnostic approach used. The advent of sequence-independent (nucleic acid-based) high-throughput technologies of microbial identification that enable microbial profiling (detection of both pathogenic and nonpathogenic microbes) in clinical specimens has permitted more precise diagnosis. *In vitro* and *in vivo* propagation of viruses also produces subgenomic viral particles (DI particles) that in some affect the phenotypic and virulence properties of heterologous viruses during coinfection, a topic still needing further investigation. Genome-wide transcriptomics and proteomics, coupled with small interfering RNA (siRNA) screens to analyze cellular factors required for virus replication, are likely to identify key molecules crucial for innate and adaptive viral interference. The approaches could also elucidate mechanisms of viral persistence, accommodation, enhancement, and superinfection exclusion/suppression during viral coinfections.

A well-adapted immune response is also critical for efficient control of pathogens involved in heterologous infections. Thus, memory responses to one infecting virus can markedly influence the type and magnitude of the immune response mounted against subsequent infections. In addition, the secondary infection may either deplete aspects of existing immune memory or generate additional effects which impact immune defense. The net outcome of heterologous immune responses could be either protection or immunopathology mediated by cross-reactivity, altered immunodominance hierarchies, or a remodeled TCR repertoire. The consequences of coinfections need to be better understood and the knowledge applied to improve diagnostics, preventative vaccines, and antiviral therapies.

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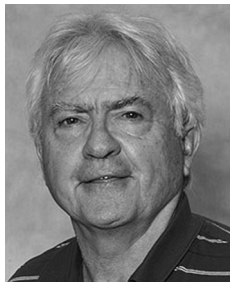

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