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Biological activities of 'noninfectious' influenza A virus particles

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Abstract

Only a small fraction of influenza A virus (IAV) particles within a viral population register as infectious by traditional infectivity assays. Despite constituting the most abundant product of influenza infection, the role that the 'noninfectious' particle fraction plays in the biology of the virus has largely been ignored. This review shines a light on this oft-ignored population by highlighting studies, both old and new, that describe the unique biological activities of these particles, and discussing what this population can tell us about the biology of IAV evolution and disease.

Keywords

aggregation; DI particles; genome packaging; influenza; noninfectious; semi-infectious; virion; heterogeneity; virus

Despite decades of effort, influenza A virus (IAV) remains a major public health burden. Seasonal strains of the virus kill tens of thousands people, and levy an estimated US$87 billion in total economic burden per year in the USA alone [1]. In addition, the potential for highly pathogenic zoonotic IAV strains to adapt to spread efficiently within the human population, triggering a catastrophic pandemic, remains both real and unpredictable. The recent unexpected emergence of pathogenic H7N9 into humans, and the surprising ease with which highly pathogenic H5N1 avian strains gain the ability to efficiently replicate and transmit in mammals in the laboratory setting, highlights the urgent need to better understand the basic biology of the virus [2–6].

The IAV genome is divided into eight discrete negative-sense, ssRNA segments that together encode at least 14 viral proteins. Expression from all eight gene segments is required for productive infection [7]. IAV infection is typically depicted in textbooks and countless presentations as a fairly orderly process involving a monolithic population of near-identical virions and a homogeneous sheet of host cells. This is a gross oversimplification, as natural infection of an animal host involves the chaotic interplay between the physically

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and genetically heterogeneous swarm of virions and a mixed collection of wildly different cell types of varying susceptibility that occurs in the midst of an incredibly dynamic and complex immune response. This complexity is a critical part of the biology of IAV infection and must be embraced in order to better predict IAV behaviour, and design next-generation vaccines and antivirals.

IAV population heterogeneity includes sequence and morphological variation, as well as variation in infectivity between particles [8–13]. Standard assays of IAV infectivity measure the number of particles capable of propagating at end point or near-end point dilution, as 50% tissue culture infectious dose (TCID50) or PFUs, respectively. Measuring the infectivity of IAV populations by these methods fails to account for the vast bulk of physical virions, as determined by hemagglutination end point or counting particles by electron microscopy (EM) [14,15]. In fact, particle-to-PFU ratios for IAV are typically greater than 10:1 [16,17]. This trait is not unique to IAV, as many other virus families also exhibit high particle-to-infectivity ratios. The reasons for this phenomenon arise from the specific biology of the particular virus, and include assembly errors, incomplete virion maturation, genome packaging failure, lethal mutation and/or the lack of fully susceptible cell culture systems [18–22]. For IAV, this phenomenon has not been studied comprehensively, and most studies focus on PFUs and ignore everything else.

I aim to shed light on the >90% of IAV virions that are usually dismissed as ‘noninfectious’. An increasing body of work makes it clear that the majority of this population is capable of infecting cells and expressing a limited, varying subset of viral proteins. Because they do not express the full set of proteins necessary for multiround replication, these semi-infectious (SI) particles do not register in traditional infectivity assays. This raises the question of the role that the SI particle majority plays in the evolutionary success of influenza virus.

The not-so-silent majority

Over the years, numerous studies have demonstrated that the noninfectious population is capable of a range of biological activities. However, investigation of this population has been hampered by difficulty in cleanly separating out noninfectious particles from normal, infectious virus [23]. Below, what is currently known about this shadowy population has been summarized.

Classic defective interfering particles

The first and, to date, still the best characterized biologically active, noninfectious form of influenza virus is the defective interfering (DI) particle, discovered as a product of infection at high multiplicity of infection (MOI) by Preben von Magnus in the 1950s [24]. DI particles were defined based on both a requirement for helper virus to propagate and the ability to interfere with the productive replication of normal virus, as determined by plaque assay [25]. The later development of an assay for DI activity based on infectious center reduction allowed for more precise quantitation of the number of DI particles within a viral population, and demonstrated that a single DI particle was sufficient to prevent plaque formation [26,27]. The relative abundance of DI particles was found to rise and fall by orders of magnitude in a predictable, cyclical pattern over repeated, high MOI passages,

with two to four undiluted passages typically resulting in DI particles outnumbering PFU by a factor of >103 [16,27–29].

It was later discovered that DI particles carry gene segments that harbor large internal deletions while retaining the 5′- and 3′-termini[30–32]. DI forms of at least seven of the eight gene segments have been observed; however, the three polymerase segments are greatly over-represented, suggesting a relationship between segment length and DI formation [32]. These DI RNA segments alone are sufficient to interfere with normal virus [33]. This explains the helper dependence of DI propagation, since all three polymerase genes are required for productive infection. However, deletion-bearing segments can be complemented by intact segments delivered by another virion [34]. It also helped to explain how the DI population expands at the expense of normal virus during high MOI passage, as deletion-bearing DI gene segments retain the packaging signals located on the 5′-and 3′-termini of the gene segments. This allows them to compete with their intact counterparts for incorporation into assembling particles[35–37].

The genesis of the deletions within DI RNAs is not understood, as the junction points between the remaining fragments are not associated with any predictable sequence motifs [23]. Sequence analysis of DI RNAs shows that the size and location of the deletions vary widely from stock to stock and tend to evolve over repeated passage[23,32]. While the *cis*-acting factors that influence DI formation remain obscure, single amino acid substitutions in either NEP (also known as NS2) or the acidic polymerase protein (PA) can enhance the production of DI RNAs [38–40]. These findings demonstrate that *trans*-acting viral genetic determinants of DI formation do exist.

The interference effect of DI particles also remains poorly characterized. DI particles do not significantly reduce the ability of normal virus to synthesize viral proteins during coinfection [28,41]. Furthermore, the observation that populations of IAV enriched for DI particles produce similar numbers of physical particles compared with normal virus shows that coinfection with DI particles does not greatly inhibit virus particle production [16,28–29]. DI particles also appear to be capable of initiating viral protein synthesis within host cells, likely resulting from primary mRNA transcription mediated by intact, virion-associated polymerase complexes [28]. The observation that coinfecting DI particles can inhibit the cytopathic effect (CPE) caused by normal virus infection under some conditions provides a possible explanation for the interference effect[16,23,42]. Because traditional assays of infectivity rely upon CPE to detect viral replication, CPE inhibition could have the same effect on the assay read-out as inhibition of replication.

Because DI particles cannot be reliably separated physically from normal virus, they are difficult to detect within mixed populations where they do not predominate. This likely explains the paucity of studies describing the *in vivo* generation of DI particles. There is good reason to expect that DI particles are produced during natural infection, as they have been found in stocks generated in cells from multiple species under a range of conditions, including low MOI passage [23]. Fulfilling this expectation, a recent study showed that the DI population expands during mouse infection [43]. Another study found sequences consistent with DI RNAs within nasopharyngeal samples from 2009 pdmH1N1-infected

humans [44]. Intriguingly, the authors found a unique DI RNA sequence, defined by deletion junction sites, that was shared between patients within the same contact network, suggesting the transmission of DI particles between individuals.

The role of DI particles during natural infection remains an open question. Nothing is known about the direct impact of DI particles on viral replication *in vivo*. DI particles limit virus-induced pathology and replication when cointroduced with normal virus into mice, but this may be through enhanced activation of the host innate immune response [43,45]. A study showed that the host cytosolic RNA sensor RIG-I preferentially binds DI RNAs rather than the longer, intact IAV gene segments [46]. Thus, DI particles may be sensed differently than normal virus and thus may influence the host immune response to IAV infection.

Other ‘noninfectious’ virion populations

Several lines of evidence indicate a substantial amount of noninterfering, infectious potential beyond that detected by standard assays. This was first suggested by a pair of studies of recombination between different temperature-sensitive (ts) mutants of the A/WSN/33 strain of IAV. In the first study, cells were coinfected with two mutants at a range of MOIs, and the frequency of recombinant progeny was determined [47]. At low MOIs, where coinfection and thus recombination should be rare, a surprisingly high frequency of recombination was observed, a result that has recently been repeated using more sophisticated techniques [48]. This indicated that a substantial amount of infectious potential was being missed by plaque assay. In the second study, the authors found that aggregates of IAV particles were 10–20-times more infectious per unit of RNA compared with single virions [49]. Together, these results are consistent with the existence of a large number of incomplete virions within IAV populations that are incapable of independent replication, but that can complement and initiate productive replication during multi-hit infection.

One obvious question that follows is whether these incomplete particles have any biological activity in the absence of complementation. An early study of an avian H7N7 strain used an *in vitro* assay of virion-associated polymerase activity to ask whether the noninfectious particle population harbored any biosynthetic capability[50]. The authors found that the amount of virion-associated polymerase activity observed within a virus population was much greater than what could be explained by the combined number of PFU and DI particles alone. This indicated that much of the ‘noninfectious’ particle population contained transcriptionally competent gene segments.

A series of papers from Marcus, Sekellick and colleagues built substantially upon these findings by quantifying the numbers of IAV particles capable of exerting different effects upon host cells. They developed an assay for quantifying particles capable of triggering cell death, in which cells are treated with virus and then plated singly to assess the reduction in colony formation. This approach revealed that the number of particles capable of killing a host cell via apoptosis is much greater than the number capable of forming a plaque for some IAV strains [51]. Thus, a large fraction of the ‘noninfectious’ particle population is capable of inducing cell death. These particles were termed ‘niCKP’ for ‘noninfectious cell killing particles’.

In addition to cell-killing activity, the authors used dose–response curves to quantify the number of IAV particles capable of inducing type-I interferon (IFN) secretion [52]. Using this method, the authors calculated that the number of particles capable of triggering IFN secretion outnumbered PFUs 10–20:1. For strains capable of efficient IFN antagonism within their system, the authors found that the number of particles capable of suppressing IFN production outnumbered PFUs by a factor of 50, again extrapolating from dose–response curves. These populations were found to be distinct from classic DI particles [16,53].

Interestingly, all three effects – cell-killing activity, IFN induction and IFN suppression – were substantially less sensitive to UV radiation than plaque-forming ability [16,52]. This is consistent with the possibility that these effects are mediated by a limited subset of the IAV gene segments rather than the full set of eight that is required for plaque formation. Together, these results demonstrate that much of the ‘noninfectious’ population is biologically active and capable of triggering cell death, and IFN induction and suppression, just like normal infectious virus.

SI particles

We recently described a novel technique for examining heterogeneity within IAV populations based on multicolor flow cytometric analysis of viral protein expression in cells infected with single IAV virions [54]. This technique allows for accurate, high-throughput examination of infectious potential at the single-particle level. Because only viral entry and protein translation are required for detection, propagation-incompetent forms of virus such as DI particles can also be analyzed. Using this approach, we observed that the vast majority of IAV virions expressed a limited subset of the viral proteins required for productive infection. This result confirmed and expanded earlier reports that viral genes were not always coexpressed at low MOI [15,55].

As might be expected, the fraction of virions that expressed an incomplete set of essential viral proteins was incapable of multiround replication in the absence of complementation. We termed this population SI, as it was capable of a single round of infection, but required complementation to propagate. For the PR8 strain, we found that the SI particles outnumbered PFUs by a factor of approximately 8, nearly accounting for all of the ‘noninfectious’ particles produced by this strain. Importantly, the ratio of SI particles to PFUs was the same for virus isolated from the respiratory tracts of infected mice and guinea pigs as it was for *in vitro*-grown virus, demonstrating that SI particle production is not an artifact of *in vitro* replication. Furthermore, SI particles of a given strain appear to be generated at a consistent frequency during every productive infection event, regardless of MOI [Brooke CB, Bennink JR, Yewdell JY, Unpublished Data]. Thus, regardless of whether incomplete particles regularly contribute to the initiation of an infection, SI particles will be the most abundant product of the first round of replication onwards.

How does the SI population relate to the other ‘noninfectious’ particle species described above? SI particles were distinguished by high-level translation of viral protein, as opposed to DI particles, which are limited in protein synthesis capacity owing to the inability to

synthesize polymerase complexes *de novo*. Thus, the SI population is separable from the DI population based on viral protein expression levels, with the exception of SI particles that fail to express polymerase components. However, the other reports of infectious or biological activity within the 'noninfectious' population are very much in line with the expected activities of SI particles.

Most SI particles should generate many of the same proapoptotic factors as normal virus, thus explaining the niCKP population [51,56]. In addition, the variable expression patterns of SI particles will result in differential effects on host cells. For example, the fractions of SI particles that do and do not express NS1 will likely differ in their interactions with different innate signaling pathways, potentially explaining the IFN-producing and -suppressing activities previously observed within the noninfectious population[52]. For PR8, the expression frequencies of the different viral gene products examined were roughly equivalent. This suggests that, in most instances, two SI particles coinfecting the same cell will complement each other's defects, resulting in productive infection. Particle aggregation, by enforcing coinfection by bound particles, could enhance PFU titers [49]. Because SI particles greatly outnumber PFUs, a dose of virus calculated to result in low MOI infection based on PFU titer actually results in a much higher effective MOI, thus explaining the unexpectedly high recombinant frequencies observed during ostensibly low MOI infection [47,48].

Most if not all of the 'noninfectious' particle population consists of either DI or SI particles. While it remains to be seen how common DI particles are outside of contrived laboratory conditions, SI particles are a normal product of IAV infection, both *in vitro* and *in vivo*. Below, I outline how and why this might be the case.

Opportunities to fail

The viability of incomplete IAV particles is made possible by the genetic architecture of the virus. Each gene segment is bound together with a copy of the trimeric RNA polymerase and numerous copies of the viral nucleoprotein (NP) into a ribonucleoprotein complex (RNP; reviewed in[57]). Each RNP is capable of acting as an independent replicative unit; thus, the absence of one functional gene segment does not necessarily prevent the expression and replication of the other gene segments [58]. This arrangement confers certain evolutionary benefits to the virus. First, it facilitates the easy recombination of segments into maximally fit combinations [59]. Second, it allows for precise regulatory control of individual viral genes, a task that is more difficult for nonsegmented RNA viruses [60].

Given the life cycle of the virus, there are a number of nonmutually exclusive explanations for the production of so many incomplete particles:

Failure to deliver entire sets of RNP s to the nucleus

Following fusion with the host cell membrane, RNPs must traffic through the cytosol and enter the nucleus in order to initiate mRNA transcription and RNA replication. The mechanisms governing nuclear trafficking are not well understood, although there is evidence that virus–host membrane fusion and RNP release following clatherin-mediated

endocytosis (CME) do not occur until virus-containing endosomes are actively transported to the perinuclear region, thus shortening the distance that RNPs must travel through the cytosol [61]. IAV has the ability to enter the cell via macropinocytosis as well, and it is not known how different routes of viral entry might affect the efficiency of RNP nuclear trafficking [62,63]. A recent study tracking individual genomic vRNA molecules postentry by single-molecule FISH (smFISH) suggested close association between the individual gene segments during cytosolic trafficking [64].

Failure to initiate gene expression following nuclear entry

The individual gene segments are delivered to the cell bound to a functional viral polymerase complex, but must also interact with numerous host factors to initiate transcription [57,65–69]. The failure rates of these various interactions are unknown and likely vary depending on host context.

Gene-lethal mutations

The error-prone IAV RNA polymerase is estimated to have a mutation rate of approximately 10^{-5} per replication cycle [12,70–71]. Spontaneous mutations can result in the selective failure to synthesize detectable levels of one or more viral proteins, due to disruption of RNA transcription or protein synthesis/folding. The predicted frequency of such mutations is nowhere close to explaining the observed expression failure rates.

Failure to copackage all eight gene segments

An extensive body of work, utilizing a range of experimental approaches, has clearly demonstrated that IAV has evolved to selectively package a single copy of each of the eight gene segments into budding particles (reviewed in [72]). Although the packaging mechanism is not fully understood, some general features have come into focus.

Following nuclear egress, RNPs traffic towards the site of particle assembly at the plasma membrane [57]. Along the way, the different gene segments interact through well-mapped packaging signals in the 5′- and 3′-termini of each vRNA segment, as well as less-well-defined sequences within the interior of some segments [72–75]. These interactions likely mediate the highly ordered ‘7+1’ arrangement of RNPs, in which a ring of seven RNPs surrounds a single central RNP, that has been observed in budding particles by scanning transmission EM (STEM)[10,73,75–77]. Chou *et al.* examined the copackaging rates of individual PR8 gene segments by performing smFISH on virus particles and reported the examined segment pairs (segment 1 with segments 2–8; segment 2 with segments 6 and 7) were copackaged in approximately 89–97% of particles [78].

Despite the structural evidence, the simplest explanation for the SI particle phenotype is failure to package one or more gene segments. Our results, generated by examining the expression profiles of hundreds of thousands of PR8 virions, indicate expression frequencies for the individual gene segments of approximately 70–80%, not far off from the lower range reported by Chou *et al.* It will require comparing different IAV strains and different mutants with known packaging defects using a combination of FACS, smFISH and EM to finally determine the contribution of packaging failure to the production of SI particles.

It is likely that all of the above listed mechanisms (and even others not listed) contribute in different measure to the limited expression patterns of SI particles.

Viral genetic control of SI particle production

In our study, we found that the ratio of SI particles to PFU varied significantly between IAV strains, and these ratios were maintained across multiple low multiplicity passages, both *in vitro* and *in vivo*. The stability of this phenotype indicates that the ratio of SI virus to normal virus produced during infection is under genetic control by the virus. The fact that some strains of IAV have evolved to produce larger relative fractions of SI virus raises the intriguing possibility that, in some contexts, increased SI virus production may be associated with increased fitness.

There are numerous viral RNA (vRNA) and protein domains known to regulate aspects of replication that could influence SI particle production. In particular, mutations within M2 or deletions of the cytosolic tails of HA or NA reduce gene segment packaging efficiency and, predictably, relative fitness [79,80]. Importantly, it does not follow that all mutations that reduce gene packaging are detrimental. It is conceivable that mutations that cause more subtle reductions in gene segment packaging, either selectively or across the board, may actually increase fitness in some contexts. Identifying and interrogating viral determinants of SI particle production will be essential in determining the role that SI virus plays in IAV replication, evolution and pathogenesis.

Are ‘noninfectious’ particles defective?

SI particles, rather than PFUs, represent the major infectious product of IAV infection. Thus, IAV should be envisioned as a swarm of functionally aneuploid particles, capable of expressing a variable, limited subset of viral proteins and dependent upon complementation to propagate. Although this is at odds with the prevailing viewpoint that the virus has evolved to maximize the production of fully infectious particles through a near perfect genome packaging mechanism, it does not necessarily imply a defect of any kind.

Segmented viruses exhibit a range of packaging strategies, from near-perfect copackaging for reovirus and 6 bacteriophage to the absence of copackaging for some plant viruses[81–84]. Low copackaging frequencies have been hypothesized to evolve when the MOI is consistently high throughout the course of infection[72]. For SI particles to contribute to the replication and spread of an IAV population, rather than serve as wasteful, dead-end products, the MOI must remain consistently high such that complementation is commonplace. The remainder of this review discusses the possibility that IAV has evolved to maintain high multiplicity throughout the course of infection.

How ‘noninfectious’ particles might contribute to infection

It was shown decades ago that IAV particles can self-aggregate, resulting in enhanced infectivity[49]. Self-aggregation is mediated by binding of hemagglutinin (HA) and sialic acid residues between particles, and can be minimized or reversed by the sialidase activity of the neuraminidase (NA) protein [85]. As a result, changes in the functional balance between

these competing activities of HA and NA can modulate the extent of aggregation, as well as interactions with host receptors [86].

In addition, the mammalian respiratory tract has numerous features that could promote virion aggregation and high MOI infection. Unlike cell monolayers or allantoic membranes, where newly released particles are able to freely diffuse into the surrounding fluid, respiratory epithelial cells are covered in a 0.5–5-μM thick layer of mucus[87]. Within and underneath this layer are a variety of constitutively expressed host factors, such as mucins and surfactants, as well as various collectins including MBL and ficolin, that are capable of binding and agglutinating IAV particles [88–93]. Together, these, and possibly other factors, likely conspire to restrict free diffusion of particles. Unsurprisingly, studies of viral spread in differentiated human airway epithelial cultures reveal a clear focal pattern of viral distribution that is consistent with preferential infection of neighboring cells [94]. While the *in vivo* burst size is not known, and will certainly vary based on many different factors, estimates range from 10 to 300 PFUs per producer cell [95,96]. Even by conservative estimates (10 PFUs ≈ 100 SI particles), this amount of virus restricted to neighboring cells would result in an enormously high MOI.

Much of the argument for why maximizing packaging fidelity would be advantageous comes from the belief that IAV goes through a low MOI bottleneck during transmission. Natural infections are initiated primarily through direct and indirect contact with virus-coated surfaces, or through inhalation of virus-bearing respiratory droplets [97–99]. Regardless of route, the transmission dose of IAV is thought to generally be low. Studies of aerosol infection in humans have estimated minimal infectious doses of <1–300 TCID50 using different human seasonal strains[100–102].

Based on these findings, many assume that the MOI on the initially infected cells is effectively 1, since the number of transmitted particles is small compared with the number of target cells in the recipient tissue. This assumption, employed to argue against a role for SI or DI particles in natural infection, fails to consider the potential for virion aggregation in respiratory droplets or aerosols, which could allow even minute numbers of particles to achieve effective MOIs of >1. While directly addressing this possibility experimentally will be difficult, recent evidence suggesting transmission of DI particles between individuals indicates that it might be relatively common [44].

If IAV infection does typically occur at high MOI, the value of attaining near-perfect gene segment packaging fidelity begins to diminish. Selectively packaging eight distinct gene segments is an extremely complex and demanding task that requires the maintenance of an extensive network of interacting protein and RNA domains, a significant cost given the limited genome size of the virus [72]. This cost could be expected to increase with increased packaging fidelity, and may simply not be worth it.

Conclusion

The existence of IAV as a population of primarily complementation-dependent, incomplete particles strongly suggests that the virus has evolved to depend upon consistent high-

multiplicity infection at all stages of the virus life cycle. This dependence likely represents a guiding factor in the adaptation of the virus to thrive in differing host conditions, including introduction into novel species and varying states of pre-existing immunity. Exploring the biology that governs SI and DI particle production, virus aggregation and *in vivo* multiplicity will be key to better understanding IAV evolution and disease.

Future perspective

First and foremost, the predominance of SI particles requires a major overhaul in thinking about influenza genetics. The effective genome of the virus is not the eight segments packaged into a single particle, but rather the collection of segments delivered to a host cell. Frequent coinfection offers the potential for novel forms of compensation as well as rampant phenotypic mixing. In addition, the dependence of most IAV particles on coinfection to propagate promotes the mixing of gene segments from multiple inputs, and is thus likely to boost the frequency of recombination. The quantity and quality of SI production will likely be important in promoting both the generation of novel genotypes during mixed infection and the generation of optimally fit combinations of variant segments during infection with a single strain [48,59].

Critical to understanding the role of SI particles in IAV infection will be identifying the viral genetic determinants that control the variation in SI particle production seen between strains. Identifying these determinants will enable experimental manipulation of the SI population, and illuminate the molecular mechanisms that drive SI versus complete virus production. Another topic of great interest is the potential for interplay between SI particle production and other facets of IAV biology, such as HA:NA balance and particle aggregation.

The *in vivo* prevalence and activity of DI particles also remains an open and important question. Further efforts to address this question, as well as identify additional genetic determinants of DI generation, should be undertaken.

Finally, the existence of IAV as a swarm of SI and DI particles mixed with a much smaller fraction of complete virus has practical consequences for how we study the virus. Current propagation-dependent assays of infectivity such as TCID50 and plaque assay are flawed because they only measure complete virus. Thus, two virus populations that have been normalized based on PFUs might actually have significantly different amounts of actual infectivity. New assays that take all forms of IAV particles into account will be key in future comparisons of different IAV strains or mutants [103].

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Executive summary

Background

- Greater than 90% of viral particles produced during influenza A virus (IAV) infection register as noninfectious by standard infectivity assays.
- The ‘noninfectious’ particle population primarily consists of two distinct types of biologically active particles that can propagate if complemented through coinfection.

Defective interfering particles

- Interfere with normal virus replication in some systems.
- Harbor large internal deletions within one or more polymerase gene segments.
- Expand during high-multiplicity infection.
- Are possibly more immune-stimulatory than normal virus.

Semi-infectious particles

- Greatly outnumber normal virus (as measured by PFUs or 50% tissue culture infectious dose).
- Do not interfere with normal virus replication.
- Fail to express one or more viral gene products.
- Produced during every IAV replication event.
- Production varies widely between IAV strains.

Implications

- The production of so many incomplete IAV particles suggests that coinfection is common throughout the course of infection.
- Coinfection may be promoted by particle aggregation and restriction of free particle diffusion *in vivo.*