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Capsid and Infectivity in Virus Detection

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Abstract The spectacular achievements and elegance of viral RNA analyses have somewhat obscured the importance of the capsid in transmission of viruses via food and water. The capsid's essential roles are protection of the RNA when the virion is outside the host cell and initiation of infection when the virion contacts a receptor on an appropriate host cell. Capsids of environmentally transmitted viruses are phenomenally durable. Fortuitous properties of the capsid include antigenicity, isoelectric point(s), sometimes hemagglutination, and perhaps others. These can potentially be used to characterize capsid changes that cause or accompany loss of viral infectivity and may be valuable in distinguishing native from inactivated virus when molecular detection methods are used.

Keywords Capsid · Epitope · Inactivation · Infectivity · Receptor

RNA Viruses Transmitted via the Environment

It is generally surmised that RNA preceded DNA in evolutionary time. The evolution of viruses becomes meaningful only after organized cells are available to be infected. At that point, the advent of capsids becomes critical, and the capsid's essential functions can be considered to include prevention of environmental degradation and facilitation of the viral RNA's entry into the host cell. Whereas the protective function is generic, the facilitation function differs, depending on whether bacterial, plant, or

animal cells are to be infected. Only enteric viruses of animals will be considered here.

These agents are typically transmitted by a fecal–oral cycle, sometimes by direct contact, but often indirectly via the environment. Agents to be considered include the picornaviruses, caliciviruses, hepatitis E virus (HEV), and astroviruses, all of which are small (~30 nm diameter), un-enveloped, and contain a single strand of plus-sense RNA. They tend to show icosahedral ($T = 1$ or $T = 3$) symmetry (Harrison 2007). Rotaviruses are also important but differ so significantly from the small RNA viruses as to preclude generalization with the others.

Vehicles of environmental transmission are food and water; some of these viruses also persist well on fomites (inanimate objects) such as floors and work surfaces. Vectors are generally mechanical (e.g., houseflies), rather than biological (e.g., mosquitoes). Indirect transmission may entail persistence of the virus in the environment for days to months under a variety of conditions. Infection will occur only if the viral RNA has retained its functional integrity (Rodriguez et al. 2009) and if the capsid, having protected the RNA to that point, is still able to attach to the receptor of a cell and initiate the process by which the RNA enters the cell.

Capsid Coding in the Viral Genomes

Coding of viral structural proteins is quite diverse, as are the capsid structures themselves. The four structural proteins of the picornaviruses are coded at the 5' translated end of the genome and are assembled in 60 copies of each per virion (Racaniello 2007); they are translated directly from the viral RNA (Ball 2007). Caliciviruses have two structural proteins, which are coded at the 3' end of the genome

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(Green 2007), as are the three structural proteins of the astroviruses (Méndez and Arias 2007) and the (probably single) structural protein of HEV (Emerson and Purcell 2007). Structural proteins of astroviruses and caliciviruses are translated from subgenomic segments of antigenomic viral RNA (Ball 2007). Comparable information is evidently not yet available for HEV (Emerson and Purcell 2007).

Capsid Functions

Essential

The essential functions of the capsid are to protect the functional integrity of the viral RNA when the virion is outside the host cell and to initiate the infectious process when a receptor on a suitable host cell is encountered. Because the capsid is outermost, most agents of inactivation must act on the capsid before they can reach the viral RNA, and in many instances inactivation entails little or no modification of the viral RNA. If the virion's capsid is functional, it affords the primary host species specificity and tissue tropism of the virus—viral RNA can infect a “foreign” cell if properly introduced, but only through a single replicative cycle: encapsidated progeny virions cannot attach to the heterologous receptors on adjacent cells. Studies indicate that a single virus type may either induce uptake of the viral RNA by the host cell or engulfment of the entire virion (pinocytosis) by the cell, with subsequent uncoating (Racaniello 2007). Initiation of infection may be inefficient, with as many as 1000 virions per plaque-forming unit (PFU), for a number of different reasons. The finding that norovirus receptors, both in vivo and in shellfish, resemble human histo-blood group antigens has many possible applications (Huang et al. 2003; Le Guyader 2006; Marionneau et al. 2002; Tian et al. 2005, 2007).

Incidental

Incidental properties of the capsid include antigenicity, isoelectric point(s), sometimes hemagglutination, and perhaps others. Epitopes on the capsid evoke antibody responses by the host organism; these epitopes are often structural, comprising overlapping portions of adjacent structural proteins. Therefore, subviral capsid proteins may not evoke antibody that reacts with the entire virion. Antigenic specificity formed the basis for differentiating virus types until RNA sequencing was made to serve this purpose more precisely. Regardless of the class of antibody, neutralizing antibody by definition prevents the virion from initiating infection at the cell level. In some, but

not all instances, the epitope may appear to coincide with the receptor-binding site on the capsid (Nuanualsuwan and Cliver 2003a). The virus is not modified by its association with the antibody, whereby the virus is restored to infectivity if the neutralizing antibody is removed by acidification or proteolytic digestion. Poliovirus neutralized by coproantibody (IgA) was reactivated by treatment with stomach or duodenal contents of pigs that had been fed a human-food diet (Cliver and Kostenbader 1979). Neutralization cannot be demonstrated with virus that lacks a laboratory host, but other modes of virus-antibody reaction (e.g., complement fixation, antigen capture, hemagglutination inhibition) may be observed. A broad-spectrum method for capturing viruses from environmental samples by means of pooled human immune serum globulin has been described (Deng and Cliver 1984). Antigenicity offers no known benefit to the virus, though it is conceivable that association with coproantibody might offer some protection against certain types of environmental inactivation.

Somewhat analogous to antibody neutralization was the reported “inactivation” of enteroviruses by grape juice, wines, and other fruit juices (Konowalchuk and Speirs 1976, 1978a, 1978b). Poliovirus inactivated by grape juice had a diminished ability to attach to host cells; when treated with stomach or duodenal contents of pigs that had been fed a human-food diet, infectivity was restored, suggesting that if ingested, such virus would be fully infectious (Cliver and Kostenbader 1979).

The proteins comprising the capsid have a collective isoelectric point, which may oscillate between two values (Mandel 1971). This oscillatory property may be lost with inactivation, which is to say that the more stable configuration is not functional in initiating infection. Not a great deal of attention is paid to viral isoelectric points in recent years, except when net charge is used to collect virions by adsorption. There is apparently no optimal isoelectric point from the standpoint of infectivity, as different types of viruses that are equally infectious may have different isoelectric points.

Some viruses (e.g., echovirus 7) are capable of agglutinating red blood cells. There is no indication that this ability plays a role in vivo, but loss of hemagglutinating ability indicates that the capsid has been degraded, and hemagglutination inhibition by antibody can be used in virus identification (Salo and Cliver 1976). Hemagglutination by noroviruses apparently does have biological significance, in that human histo-blood group antigens appear to resemble the cell receptors to which the viruses attach to initiate infection (Hutson et al. 2003).

There may well be other fortuitous properties of the virion that could be used to determine the integrity of the capsid. One that has been exploited is resistance to digestion by proteinase K (Nuanualsuwan and Cliver 2002).

Inactivation Events Involving the Capsid

As was indicated earlier, virus is no longer infectious when the capsid loses its ability to protect the RNA from environmental degradation or to initiate infection on contact with the receptor of an appropriate host cell. The distinction between infectious and inactivated virus has great significance in testing vehicles and fomites, but probably none when testing clinical samples from a patient. Various measures are imposed to inactivate viruses, either specifically (e.g., chlorination) or fortuitously (e.g., cooking): the success of these measures is hard to judge with viruses that have no laboratory host, and the determination is slow and costly when laboratory hosts and their homologous viruses are employed for the purpose (Rodriguez et al. 2009). After inactivation at 37°C, poliovirus 1, hepatitis A virus (HAV), and feline calicivirus were shown to have lost affinity for homologous host cells (Nuanualsuwan and Cliver 2003a); the viral RNA was apparently intact. Viruses for which cultured host cells are available may be shown, by means of a molecular beacon, to have penetrated and uncoated in no more than 2 h (Yeh et al. 2008a, b); the method may not yet have been applied specifically to demonstrating functional degradation of the capsid.

Agents of virus inactivation may be chemical, physical, or biological. Chemical agents include strong acids, strong alkalis, strong oxidizing agents, alcohols, formaldehyde, etc. Physical agents include heat (time–temperature), drying, and electromagnetic radiation. Biological inactivation is based on attack of the capsid by enzymes or other microbial products.

Chemical Inactivation

Enteric viruses are categorically resistant to pH 3, and HAV to even lower pH (Hollinger and Emerson 2007; Green 2007); this purports to relate to ability to withstand stomach acidity, but it should be noted that some bacteria that infect perorally with high efficiency (e.g., *Salmonella*) are quite acid-labile. High pH, on the other hand, is poorly tolerated; pH effects are contingent on the presence of other solutes and on temperature (Salo and Cliver 1976). Inactivation results in alterations to the physical integrity of enteroviruses. At pH 5 and 7, RNA hydrolysis of poliovirus particles occurs; and at pH 3, 5, 6, and 7 the nucleic acid becomes susceptible to RNase. Only virus particles inactivated at pH 3 showed sensitivity to chymotrypsin.

Strong oxidizing agents, such as chlorine, attack both the capsid and the RNA; but under some circumstances the RNA may still give a positive RT–PCR result after the capsid has been functionally degraded (Nuanualsuwan and Cliver 2002). Although the chlorine-inactivated virus gave a positive RT–PCR result, its RNA lost infectivity at

essentially the same rate as the whole virion (Nuanualsuwan and Cliver 2003b). Part of why RT–PCR results may remain positive with poliovirus inactivated by ClO₂ is that the most labile portion of the genome is in the 5′-non-translated region, which is seldom targeted in RT–PCR testing (Simonet and Gantzer 2006), so the disparity need not result from capsid degradation—which was not tested. Duizer et al. 2004 reported that norovirus inactivation (estimated indirectly) by chlorine, but also by UV, extreme pH, or heat, was not accompanied by negative RT–PCR results, though quantitative RT–PCR sometimes showed significant changes. On the other hand, ozone disinfection of norovirus was said to produce parallel decreases in estimated infectivity titer and RT–PCR signal (Shin and Sobsey 2003). More studies are needed on how the capsid is affected during chemical inactivation.

Physical Inactivation

High temperatures are clearly detrimental to capsids—temperatures of 95°C or higher are sometimes used as a means of extracting RNA from viruses, so the RNA is the more heat-stable moiety. In the case of poliovirus, heating results in loss of all 60 copies of VP4, with concomitant change in antigenicity and loss of ability to attach to host cell receptors (Pallansch and Roos 2007). VP4 is a very small peptide in HAV and is not detectable in the fully encapsidated virion, which may partly explain why HAV is more heat-stable than polioviruses (Hollinger and Emerson 2007). Inactivation at 37°C results in loss of ability to attach to host cell receptors, without loss of the protective function of the capsid (Nuanualsuwan and Cliver 2003a). Like other chemical reactions, chemical disinfection is likely to be accelerated with increased temperature.

Enteroviruses are generally inactivated by drying, whereas HAV and noroviruses are not. This has an important influence on transmission—especially where fomites are involved.

Electromagnetic radiation may be either ionizing or nonionizing. Ionizing radiation (⁶⁰Co γ-rays) had to be applied at relatively high doses to inactivate a variety of viruses in aqueous suspensions, due to their small target size (Sullivan et al. 1971). Coxsackievirus B2 required higher doses for inactivation in ground beef than in aqueous suspensions, presumably because the ground beef scavenged free radicals that would otherwise have attacked the virus capsid (Sullivan et al. 1973). Nonionizing (UV) irradiation is often used to decontaminate water, wastewater, and surfaces, although it cannot penetrate foods or turbid aqueous suspensions. UV-inactivated poliovirus 1 was unable to attach to homologous cell receptors or to react with homologous antibody, whereas UV-inactivated HAV was still partially able to attach to cell receptors and

was captured by homologous antibody (Nuanualsuwan and Cliver 2003a).

Biological Inactivation

Enzymes and perhaps other products of microbial origin have been shown to attack viruses (Deng and Cliver 1995). Some of the proteolytic enzymes were partly characterized, but their precise effects on the viral capsid were not. Trypsin-cleaved poliovirus did not lose infectivity or antigenicity (Fricks et al. 1985). The antiviral activity of bacteria is exploited in the activated sludge treatment of wastewater (Ward 1982).

Exploitation of Capsid Changes to Avoid False-Positive Detection Results

It seems clear that essentially every mode of inactivation has some perceptible effect upon the capsid. Therefore, it is reasonable to seek means of exploiting these changes to eliminate false-positive results in RT–PCR testing.

Capture

In instances where the capsid of the inactivated virion loses its antigenicity, failure of antibody capture might afford a negative test result. One recent technique, erroneously called “immunomagnetic capture,” does not use antibody and may not offer this distinction (Hirneisen et al. 2009).

As more is learned about the early interaction between the capsid and cell receptors (Bubeck et al. 2005; Fricks and Hogle 1990; Kaplan et al. 1996), it may be possible to exploit the inability of inactivated virus to attach to these receptors (Nuanualsuwan and Cliver 2003a). The identification of human histo-blood group antigens as receptors for noroviruses offers many possibilities in this regard (Huang et al. 2003, 2005; Hutson et al. 2003).

Degradation

Since the capsid is protein, it is reasonable to begin with proteases as means to degrade the compromised capsid: we began with chymotrypsin (Salo and Cliver 1976) and changed to proteinase K (Nuanualsuwan and Cliver 2002), but neither of these sufficiently degrades capsids affected by all of the tested inactivating agents, to expose the viral RNA to RNase. Many further modes of inactivation have not been investigated from this standpoint. Not long before my retirement, we set about screening 10 proteases of animal and vegetable origin that appeared relatively inexpensive and easy to handle; unfortunately, we encountered some confounding variables and ran out of time. There are

many proteases that meet the “cheap and easy” criteria: hopefully the most problematic mode of inactivation could be selected for screening. In our hands, proteases were particularly challenged by viruses inactivated at 37°C, but lower temperatures or other modes of inactivation may be more problematic still. There is also the possibility that no one mode of inactivation will afford the perfect screening tool. UV or heat inactivation of HAV has been reported to diminish the RT–PCR signal without application of protease or RNase, especially if the primer set targets the 5' end of the viral genome (Bhattacharya et al. 2004); but the RT–PCR signal of heat-inactivated murine norovirus could not be completely eliminated, even with combined treatment with proteinase K and RNase (Baert et al. 2008).

On the other hand, it may not be necessary to restrict the quest to proteases. Enteric viruses are notoriously resistant to chemical challenges, such as extreme acidity or organic solvents; losses of either of these classes of protection might be tested. The finding that RNase remained functional when mixed with proteinase K was fortuitous: two-step treatments, with RNase applied subsequently, are certainly feasible, but some of the protease alternatives might obviate the use of RNase entirely. Detection of norovirus capsid by mass spectrometry might focus directly on capsid degradation in inactivation (Colquhoun et al. 2006).

It is also noteworthy that the hypothetical treatment to eliminate false positives may not always need to be universal. One might be concerned with the effectiveness of a specific disinfectant, in which case the treatment should work with several viruses inactivated by this one agent. Otherwise, one might target a specific virus or group of viruses on the basis of the primer sets to be used in RT–PCR, since false positives are not an issue with the viruses that the molecular test is not designed to detect.

Conclusions

Thus far, every mode of inactivation affecting small, round enteric RNA viruses has been shown to entail some demonstrable modification of the capsid. Infectivity or lack thereof is not an issue with clinical samples, in that the diagnosis is likely to be straightforward. On the other hand, samples of food, water, etc., that give a positive RT–PCR test result with inactivated virus are likely to be perceived as threats to public health where none exists. The ideal solution would involve a single, simple pretreatment of a sample that would quickly preclude RNA amplification from non-infectious viruses. Alternately, a treatment that was specific for one group of viruses with all means of inactivation or for one means of inactivation with all groups of viruses would be very useful. Least desirable, but still useful, would be a

treatment specific to a single mode of inactivation and a constrained group of viruses; even this might be applicable in designing processes needed to eliminate a known, high-risk virus in a specific context. The main point is that, in all of these applications, the capsid is key.

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