MOLECULAR ASPECTS OF HYPOVIRULENCE: STATE OF THE ART

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ABSTRACT. *--Transmissible hypovirulence has been consistently associated with the presence of one or more components of high molecular weight dsRNA. Unfortunately the direct proof, by cell-free transmission, that the dsRNA is the hypovirulent factor is still lacking. Evidence would suggest that this dsRNA may be a biologically unique* entity *because it lacks a protein capsid. It is also unique because the numbers of dsRNA components present may be variable. Nothing is known of the relationship of the dsRNA of one strain to that of another. There are both transmissible and non-transmissible forms of hypovirulence of E. parasitica. While there are undoubtedly many forms of non-transmissible hypovirulence, there may be biochemical or genetic simularities between transmissible and some types of non-transmissible hypovirulence. A better knowledge of the genetics and control of virulence of E. parasitica is needed to understand how dsRNA or other factors may reduce the virulence of this pathogen. Fortunately, the fact that virulence of E. parasitica can be perturbed in a number of ways can be utilized to better understand the genetics of virulence and hypovirulence.*

Hypovirulence of *Endothia parasitica* has caught the imagination of people because it offers hope for the restoration of the American chestnut. As a result, most of the research concerning hypovirulence has justifiably emphasized field applications of this biological control phenomenon. The molecular aspects of hypovirulence, however, are equally exciting. The control of virulence expression in a pathogen by cytoplasmic genes has been an area of rapid and exciting discovery. For instance, it has been found that plant tumor induction caused by *Agrobacterium tumefaciens* (Merl() 1978) and *Pseudomonas savastanoii* (Comai and Kosuge 1980) are controlled by plasmid genes. Until the discovery of hypovirulence of *E. parasitica,* however, all of the known transmissible virulence control systems acted to increase the virulence of the pathogen. Hypovirulence of *E. parasitica* is unique because the cytoplasmically transmissible genes reduce pathogen virulence. Study of this system may provide researchers with clues concerning the genetic control of virulence and perhaps point the way to creation of similar hypovirulence control systems in other pathogens.

These research goals are naturally speculative and their realization years ahead. For the immediate goal of improving field spread of hypovirulence, knowledge of the molecular aspects of hypovirulence of *E. parasitica* is imperative. Our current knowledge of the nature of these cytoplasmically

transmissible hypovirulence control genes is very limited. To understand how they may spread and the limitations to their spread we must first know something of their biological nature. There is good correlative evidence that the hypovirulence factor genome is encoded on double-stranded (dsRNA). Direct evidence for this is needed however. We must also determine how the dsRNA is packaged within the fungal cell since this may be a critical factor in determining how spread occurs, or is limited. Finally, there has been much speculation concerning the origin and possibility that we are dealing with more than one hypovirulence factor. These questions cannot be answered without knowing much more about the genome and packaging of the hypovirulence factor.

In this short discussion of the molecular aspects of hypovirulence we have identified four different questions that we feel should be addressed as an initial step in understanding this phenomenon. The first three are very basic questions concerning the nature of the hypovirulence factor: 1) Is dsRNA the genome of transmissible hypovirulence? 2) Is the dsRNA associated with hypovirulence the genome of a virus? 3) What is the nature of the dsRNA associated with hypovirulence? The fourth question is: How does the dsRNA regulate the virulence of *E. parasitica?* This last question is not likely to yield answers as readily, in response to a researcher's efforts, as the first three. It is an important one, however, since it may hold the key to the success of expanding hypovirulence to other systems. We have organized our discussion to address what is currently known about each of these questions, and to make suggestions concerning what we need to know.

Is dsRNA the Genome of Transmissible Hypovirulence?

It is universally accepted among those working with hypovirulence of *E. parasitica* that the dsRNA extracted from hypovirulent strains is responsible for the control of hypovirulence expression. Unfortunately, the evidence for this is all correlative, and thus the conclusion so universally accepted may be wrong. There is a need for direct evidence of the role of dsRNA in hypovirulence, such as the cell-free transfer of dsRNA into virulent cells with the resultant transfer of the hypovirulence phenotype. One of the reasons for the confidence in the correlative evidence is that it is good. It has been shown that 1) as dsRNA is carried with the cytoplasm of a transmissible hypovirulent (TH) strain into a virulent (V) one during hyphal anastomosis, the TH phenotype follows the dsRNA (Anagnostakis and Day 1979), 2) as dsRNA is lost from a TH strain, the TH phenotype is also lost (Van Alfen and Gillies unpublished data), and 3) specific TH phenotypes associated with specific dsRNA segments follow those segments upon transmission (Elliston 1981). At least one of the isolates (EP-49) that contains dsRNA has been judged to have comparable virulence to some isolates that do not contain dsRNA (Elliston 1978). This would seem to negate the correlative evidence between hypovirulence and dsRNA. However, some of the conidia obtained from this isolate are typically hypovirulent and contain dsRNA (Van Alfen et al. 1978a). Thus, the presence of dsRNA within the thallus of this isolate can be correlated with the potential of producing typical TH progeny.

One of the problems with correlative evidence is that subjective judgments must be made. Since virulence in *E. parasitica* is acontinuum (Elliston 1978),

there is a point where a line must be drawn to distinguish virulent from hypovirulent isolates. One is naturally inclined to draw that line between TH and V at the point where dsRNA is no longer found. Direct evidence would reduce the possibility that the prejudice of the investigator is influencing the conclusion.

Cell-free infection of V strains by the dsRNA if simple, would have been accomplished by now. It has been attempted in more than one laboratory. Even the simpler problem of cell-free infection of fungi by intact mycoviruses has been successfully reported only a few times (Ghabrial 1980). The advantage mycoviruses would have over naked dsRNA for cell-free infection is that mycoviruses are known to carry the RNA polymerases necessary for their replication (Buck 1980). All dsRNA mycoviruses or dsRNA alone require such enzymes for replication. Cell-free infection with phenol extracted dsRNA may thus be an impossible task since any associated RNA polymerases would be removed by the phenol. Initial replication of the dsRNA would be dependent upon enzymes present in the fungus. Knowledge of how the dsRNA is packaged and whether RNA polymerases are present within the package may help facilitate cell-free transfer to fungal protoplasts.

In our laboratory we are attempting cell-free infection by packaging the dsRNA within artificial membrane liposomes for transport of the dsRNA into the protoplast of virulent strains (Van Alfen and Hansen 1982). We are also attempting cell-free infection of protoplasts using the membrane-bound vesicles containing dsRNA that can be extracted from TH strains (Dodds 1980a). These dsRNA containing vesicles are fused with protoplasts using the same methods as employed for artificial membrane liposomes. Hopefully, one of these methods of introducing the cell-free preparation of dsRNA into virulent strains will be successful.

Is dsRNA Contained in a Virus-Like Particle?

The possibility of the dsRNA associated with hypovirulence being packaged in a virus-like particle (VLP) is not unlikely and has been suggested by many workers. Such a VLP has been reported by Dodds (1980a) and Chmelo (this proceedings). Dodds (1980a) has isolated a club-shaped particle from *E. parasitica* strain 113 which he believed packages the dsRNA within the fungal cell. This pleomorphic club-shaped particle can be extracted with polyethylene glycol and purified by differential, then cesium chloride (CsC1) equilibrium density centrifugation. In our lab, we (Hansen, Gillies and Van Alf en, unpublished data) have confirmed Dodds' observations concerning the fact that the crude preparation of pleomorphic particles can be separated into two peaks from a CsC1 gradient. The denser of the two (1.27 g/cm^3) contains the dsRNA. We have analyzed the composition of these particles and determined that they are very unlike a typical virus. There is an unusual amount of carbohydrate present. In fact, it comprises the greatest proportion of the particle. Gas chromatography analyses by the alditol-acetate method (Jones and Albersheim 1972) show the same sugars are present in the particle as those found in the fungal cell wall. Radiolabeling experiments show several different proteins are present, but not one in an amount sufficient to be a capsid. Another peculiarity about this so-called VLP is that of those strains of *E. parasitica* examined thus far, only EP-113 and EP-43 (Chmelo this proceedings) are found to contain the particles. Thus, it is

not clear whether this particle is actually a virus or something else, e.g. a fungal vesicle used to isolate the dsRNA or perhaps an artifact of the purification method.

Areas in need of future investigation are clearly evident. How is this club-shaped particle formed? If it is a virus, how and where does it replicate? Are there polymerases present? Does it have a capsid and how is dsRNA packaged within the virion? In addition, how is it related to other mycoviruses? If it is not a virus, then what is the purpose of the particle which contains dsRNA? The fact that the particle composition is similar to the cell wall may indicate that the membranes are a mode of removing dsRNA from the fungal cells. Transmission electron micrographs show VLP's associated with the endoplasmic reticulum of a hypovirulent strain, yet absent from a virulent one (Newhouse et al. this proceedings). The endoplasmic reticulum is the site of cell wall carbohydrate synthesis. This may explain the similarity between sugar composition of the pleomorphic dsRNA containing membrane-bound particles and the cell wall. These particles may be vesicles associated with cell wall synthesis. The knowledge of how the dsRNA is packaged within the fungal cell will undoubtedly help us understand variables involved in cell to cell transmission of the dsRNA.

What is the Nature of the dsRNA of TH Strains?

The dsRNA associated with hypovirulence, while the subject of much speculation, has been poorly characterized. The studies that have been completed on electrophoretic banding patterns and dsRNA size are a good beginning at characterizing the dsRNA, but they are only a beginning. We currently know that the dsRNA of different TH isolates may differ greatly in their electrophoretic banding patterns (Dodds 1980b). These banding patterns change at times, however, upon transfer to new V strains or upon subculturing (Anagnostakis and Day 1979). Such variability in the composition of dsRNA segments is not typical of the dsRNA of mycoviruses (Buck 1980). The dsRNA segments of known mycoviruses are quite stable in their electrophoretic banding patterns. Thus, we need to determine whether this variability represents mixed infections of different mycoviruses, or whether deletions from individual dsRNA segments are a frequent occurrence. A similar complex and variable segment pattern in *Gaeumannomyces graminis* var. *tritici* was explained on the basis of mixed infections (Buck et al. 1981). In the case of TH of *E. parasitica* such mixed infections may not be the explanation since dsRNA containing virulent strains have not been found (Elliston 1978). We would expect that not all dsRNA segments would cause the hypovirulence phenotype, thus mixed infections would be expected to result in the occurence of dsRNA containing V strains. Deletions in dsRNA virus genomes have been recorded, but they serve as poor models for what is occurring in the dsRNA of *E. parasitica* since deletions are a rare event (Rubenstein and Harley 1978).

Probably the most direct approach to studying the relationship between dsRNA segments found in *E. parasitica* is to determine the relatedness of the different segments to one another. Such studies can be done using hybridization techniques or nucleotide mapping. The study of the homology between the dsRNA segments would also answer questions of whether there are great differences between the dsRNA of European and North American TH strains. If

the dsRNA of the European and American TH strains is different then we must seriously consider the possibility that there are different TH factors.

In addition to homology studies, better physical characterization of the dsRNA is needed. Currently the double-strandedness of the RNA is known by behavior on cellulose columns and by selective degradation by enzymes. Physical evidence such as melting to the predicted molecular weights of the single strands is needed to confirm the double-strandedness of the RNA. Molecular weights also need to be confirmed by physical methods. Currently the molecular weights are estimates based upon comparative electrophoresis with standards (Dodds 1980b). Unfortunately the largest dsRNA segments from *E. parasitica* are at the limit of judicious use of standards.

How does the dsRNA Regulate Virulence of *Endothia parasitica?*

Virulence reduction in *E. parasitica* induced by dsRNA has no models in biology to draw upon for comparison. We currently know nothing of how virulence is modulated by the dsRNA so all we can do in this paper is speculate. There are at least three possible general mechanisms whereby the dsRNA could modulate virulence. One would be to compete by replication for critical cellular metabolites. Since most mycoviruses are latent, having no discernible effects on the fungus, replication of a mycovirus does not appear to adversely affect most fungi. However, the competition for a specific metabolite important in virulence may have an effect on virulence. Virulence could also be reduced by the effect of a translational product of the dsRNA at some metabolic control pathway critical to virulence expression. A third possibility is that the dsRNA directly interacts with the fungal genome at a virulence control locus. The existence of such a locus is suggested by the high frequency appearance of non-transmissible hypovirulent isolates after UV irradiation (Van Alfen et al. 1978b) or osmotic shock (Van Alfen and Hansen this proceedings). These non-TH isolates are phenotypically identical to TH isolates, but they lack dsRNA (Van Alfen and Hansen unpublished data). These isolates may represent a class of control site mutants.

The problem of determining how virulence is affected by the dsRNA will not be easily solved. The question does have much appeal so hopefully many researchers will be attracted to the problem to ensure progress to its solution. If a mechanism is found that is exploitable in other pathogens, the effort expended on this research could result in wide application.

Conclusion

The research questions posed in this discussion are certainly not comprehensive, nor will their solution provide more than a good beginning toward understanding the phenomenon of hypovirulence of *E. parasitica.* Progress must be made toward their solution, however, if we are to determine the potential of this system for wider exploitation. We must also learn more of the biological nature of the TH factor so that some of the problems encountered in its field use for control of chestnut blight can be overcome. Such knowledge would allow intelligent planning rather than wild guessing to direct our research efforts. Without an understanding of the properties of the TH factor we are no better able to exploit it for control of chestnut blight than we can repair our own laboratory instruments. We must have an

understanding of how our instruments work before we can repair them. Likewise we must have an understanding of the properties of the TH factor before we can optimize its spread.

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