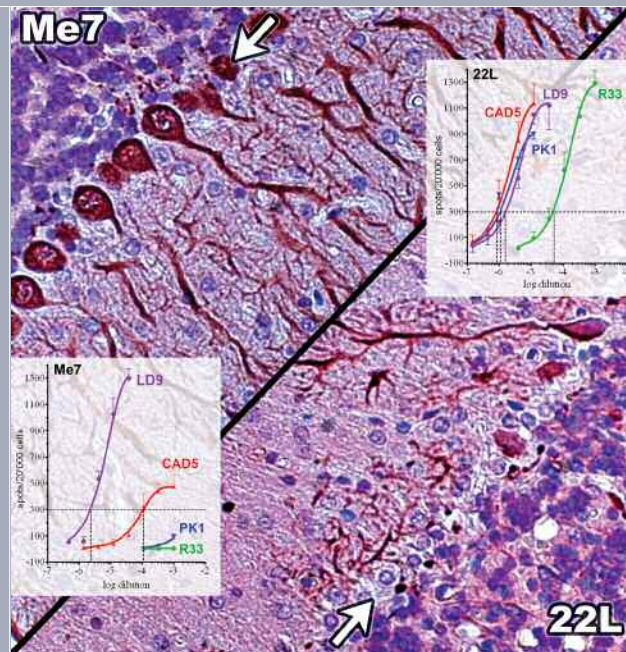


Infectology

Distinguishing between prion strains 22L and Me7 with the mouse bioassay takes 6 months. The cerebellar Purkinje cell layer (immunostained for calbindin) remains intact in Me7-infected, terminally sick mice (top, arrow) but is obliterated by infection with 22L (bottom, arrow). With the cell panel assay (insets), the strains can be distinguished from each other in 2 weeks. 22L prions efficiently infect the 4 cell lines constituting the panel, whereas Me7 infects only LD9 and CAD cells. Stained sections and micrographs prepared by Sukhvir Mahal, Ph.D., staff scientist, and Alexandra Sherman, research assistant; photomontage created by Christopher A. Baker, Ph.D., research associate. Work done in the laboratory of Charles Weissmann, Ph.D.





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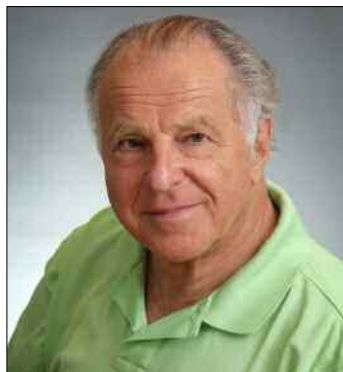
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Chairman's Overview

The Department of Infectology focuses on prion diseases and hepatitis. Tim Tellinghuisen and his colleagues study hepatitis C virus (HCV) RNA replication and virion assembly. They identified the viral protein NS5A as an essential component of the viral replicase and mapped all amino acids in domains II and III essential for HCV RNA replication. They identified regions whose functions are required for generating infectious virus but not for RNA replication, suggesting that NS5A regulates the switch between RNA replication and virus assembly. They are using high-throughput genetic screens to identify host components required for replicase activity.

Donny Strosberg and his colleagues established for the first time a cell culture system for HCV genotype 1b. They identified peptides derived from the HCV core that inhibit (1) dimerization of core protein, the first step in viral assembly and (2) release of virions. F.V. Chisari and his group, Department of Molecular and Experimental Medicine, independently found that one such peptide inhibits HCV replication. These findings establish the HCV core as a target for the development of anti-HCV drugs.

Three groups, those of Corinne Lasmézas and Joaquin Castilla and my own, study prion biology. Prions consist



Charles Weissmann, M.D., Ph.D.

of a multimeric assembly of PrP^{Sc}, a conformer of the normal host protein PrP^C. The seeding hypothesis posits that prions replicate by recruiting host PrP^C into the PrP^{Sc} assembly, a process that entails conformational rearrangement of the PrP^C. Prions occur in the form of different strains, all associated with the same PrP^{Sc} sequence but with distinct cell tropisms, both in brain and in cell culture. The cell-based assay for prions has been further streamlined by Emery Smith, and the cell panel assay, which allows rapid distinction between prion strains, was extended to more strains by Sukhvir Mahal. It has been proposed that “strain-ness” is encoded either by distinct conformations of PrP^{Sc} or by the complex glycans present on the protein. This hypothesis has been negated by Shawn Browning and Dr. Mahal, who found that strain specificity is maintained when prions are propagated under conditions in which complex glycosylation is abrogated.

Dr. Lasmézas and her colleagues determined that PrP^{Sc} in cultured prion-infected cells is undetectable at the outer cell surface and is therefore likely generated intracellularly. These investigators characterized a rapid animal model for prion disease based on a transgenic, PrP-overexpressing mouse strain and are researching the mechanism of pathogenesis. Dr. Castilla and his group have perfected the protein misfolding cyclic amplification procedure, which allows the cell-free replication of prions, and have shown that various prion strains can be propagated continuously without losing strain-specific properties.

Investigators' Reports

Biology of Prion Strains

C. Weissmann, C.A. Baker, S. Browning, C. Demczyk, M. Herva-Moyana, J. Li, S.P. Mahal, A. Oelschlegel, A. Sherman, E. Smith, I. Suponitsky-Kroyter

Prions are thought to consist mainly or entirely of PrP^{Sc}, an abnormal conformer of a normal host protein, PrP^C, and to propagate via a PrP^{Sc}-catalyzed conversion of PrP^C. Intriguingly, distinct prion strains, which generate different disease phenotypes, are associated with the same PrP sequence, suggesting that the phenotypes are encoded posttranslationally. Our major interests are the mechanism of prion replication, the structural basis of strain specificity, and the mechanism of strain recognition by cells.

Much of our research depends on a cell-based assay for prion infectivity, the standard scrapie cell assay. Therefore, considerable effort has gone into automating the procedure, reducing its cost, and improving its accuracy. We can now assay about 1000 samples per week in sextuplicate, orders of magnitude greater than the number that could be processed by using the classical mouse bioassay. The standard scrapie cell assay is the basis for the cell panel assay (CPA), which makes use of the finding that different cell lines have distinctive susceptibilities to various prion strains. Using the CPA, we can distinguish between various prion strains within 2 weeks, as opposed to the year or more required with the classical mouse bioassay. The panel originally consisted of 4 cell lines; however, when exposed to swainsonine (an α -mannosidase II inhibitor that modifies the glycans of *N*-glycosylated proteins), the PK1 cell line (but none of the others) becomes resistant to some prion strains but not to others. Therefore, swainsonine-treated PK1 cells provide an additional criterion for discriminating prion strains. We can currently distinguish 5 groups of strains (Table 1).

We determined that the characteristic CPA responses of these strains are the same whether the strains are propagated in wild-type mice or in *Tga20* mice, which overexpress PrP^C and, conveniently, have a reduced incubation time.

We have established a large collection of cell lines chronically infected with various prion strains. Interestingly, in many instances, the cell tropism of prions derived from such lines, as determined by using the

Table 1. Susceptibility of cell lines to various prion strains.*

Cell line	Prion strain				
	22L	139A	RML, 79A	Me7	301C
LD9 (EMEM)	+++	+++	+++	+++	-
CAD5*	+++	+++	+++	-	+++
PK1	+++	+++	+++	-	-
PK1/swa	+++	++	-	-	-
R33	++	-	-	-	-

* The number of pluses indicates the degree of susceptibility to infection. The minus sign indicates resistance to infection.

CPA, differs from that of the prions derived from brain. To determine whether this change reflects a permanent modification of a strain, we inoculated cell-derived prions into mice; CPA analysis of the infected brains showed no differences between the original and the cell-passaged strains. We are currently considering the possibility that the cell tropism of a prion strain may depend on the host cell in which the strain is generated, reflecting some host-imparted property (the "cytotype"), for example, the glycosylation pattern of the PrP.

It has been proposed that the *N*-linked complex glycans attached to PrP might encode the "strain-ness" of prions. To address this question, we propagated RML, 22L, and Me7 prions in CAD5 cells in the presence of the glycosylation inhibitors deoxymannojirimycin and swainsonine. These inhibitors prevent processing of the precursor of *N*-linked glycans and result in PrP with [mannose]₅[*N*-acetylglucosamine]₂ as the only glycan, rather than a multiplicity of complex sugars. We injected cell lysates of the drug-treated, prion-infected cells into mice and used the CPA to analyze the infected brains. In all instances, the strain-specific properties had been retained, proving that complex glycosylation was not the strain-determining element and adding weight to the hypothesis that strain-ness is encoded by the conformation of PrP^{Sc}.

In another project, we are exploring why certain subclones derived from the same cell line, occurring with a frequency of 1 in 5000 to 1 in 10,000, can vary by 100 to 1000 times in their susceptibility to prions. We tagged highly susceptible and resistant subclones with 2 different markers of antibiotic resistance and fused the cells. Currently, we are determining whether susceptibility, as measured in heterokaryons,

is a dominant or a recessive property. In the next step, we will generate microcells containing single, tagged chromosomes from one of the cell lines and fuse the microcells to cells from the other line to determine which chromosome encodes the critical trait.

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Pathogenesis of Transmissible Spongiform Encephalopathies

C.I. Lasmézas, N. Salès, P. Saá Prieto, M. Zhou, Y. Karapetyan, F. Sferrazza, G. Ottenberg

P rions, the transmissible agents responsible for prion diseases, are thought to consist mainly of PrP^{Sc}, an abnormally folded isoform of the ubiquitous prion protein PrP. Prions are thought to replicate by an autocatalytic process of template-induced conformational change.

In collaboration with C. Weissmann, Department of Infectology, we are devising a new method for propagating and characterizing different strains of prions. Dr. Weissmann and his group have developed a cell-based infectivity assay, the cell panel assay, in which prion strains are distinguished on the basis of cell tropism. We have studied the fate of prions in the *Tga20* mouse model. Compared with wild-type mice, *Tga20* mice express 8-fold higher levels of PrP^C in the brain, and clinical disease occurs more quickly after inoculation of prions.

Many aspects of prion replication in *Tga20* mice were unknown, for instance, the response of these animals to different prion strains. We found that PrP^C from the brains of *Tga20* mice has a higher intrinsic resistance to proteolytic digestion by protease than does PrP^C from the brains of wild-type mice. We also discovered that the levels of PrP^C overexpression in different regions of the brain vary and hence could influence the pathologic lesions and PrP^{Sc} distribution that occur after prion infection. Vacuolation and PrP^{Sc} deposition pro-

files in brains of *Tga20* mice differed from those in C57BL/6 mice for the 3 scrapie strain studied. Each strain had a characteristic profile in *Tga20* mice and could be readily distinguished by neuropathologic analysis, showing that the level of PrP^C expression is not the main determinant of brain tropism. Importantly, despite generating different neuropathologic phenotypes in C57BL/6 and *Tga20* mice, all 3 prion strains retained their intrinsic identity after being replicated in either mouse line, as determined by the cell panel assay.

This study provides a new reliable method for rapid propagation and characterization of prion strains by using a combination of *Tga20* mice and the cell panel assay. Fundamentally, our results indicate that despite different biochemical characteristics of PrP^C, different expression patterns of PrP^C in the 2 murine hosts, and different genetic background of the 2 mouse strains, prions are propagated faithfully in both types of mice. This finding raises once more the question of the molecular basis of prion-strain properties.

Previously, we found that oligomeric assemblies of recombinant prion protein are toxic to primary cultures of cortical neurons and in mice when injected via a stereotaxic method. We are now devising new intervention strategies to block the toxic/infectious PrP species. A first goal is to determine the cellular location of PrP aggregates, in order to know which cellular compartment to target. The many reports of the presence of protease-resistant PrP (PrPres) at the cell membrane are contradictory. Using a cell-surface biotinylation strategy and comprehensive controls to account for the presence of dead cells and for the intrinsic capacity of PrPres to be biotinylated, we found no detectable PrPres at the cell membrane. This finding has major implications for the development of diagnostic molecules. We are continuing our efforts to locate the cellular compartment that must be targeted for therapeutic purposes, and we are setting up new models for PrP-induced toxic effects in cell lines.

Inhibitors of Protein-Protein Interactions in Hepatitis C

A.D. Strosberg, C. Coito, R. Henderson, S. Kota, G. Mousseau

S everal small-molecule drugs are in advanced clinical development for the treatment of hepatitis C, a situation that may affect the 170 mil-

lion carriers of hepatitis C virus (HCV) worldwide, including more than 3 million in the United States. Because of its high mutability, HCV likely will become resistant to these potential drugs, which are mostly inhibitors of a viral protease and the viral polymerase. This past year, we continued our studies of protein interactions involving HCV proteins to better understand the respective roles of the proteins and to identify novel target proteins that would not induce resistance. The core HCV protein, which is highly conserved across all 6 major HCV genotypes, is a good candidate for such studies.

HCV CORE PROTEIN AS A TARGET

The HCV core protein functions primarily as the structural element of the virus. The core contains several residues essential for HCV production. Most of these residues are located in the N-terminal two-thirds of the core protein and mediate core dimerization and most interactions with intracellular proteins. Core dimerization is the first step in nucleocapsid assembly; its inhibition should block formation and release of infectious HCV particles.

ASSAYS FOR HCV CORE DIMERIZATION

Using a pair of domains that consist of the first 106 residues of the HCV core protein (core106) tagged with oligonucleotides encoding the octapeptide Flag or glutathione-S-transferase (GST), we developed an assay based on the use of an antibody to Flag that binds to Flag-tagged core106 interacting with GST-tagged core106 adsorbed on a glutathione-coated microtiter plate. We designed a 384 well-based sensitive and high-throughput time-resolved fluorescence assay with fluorescent antibodies to Flag and GST. Untagged core106 completely inhibits core106 dimerization. One of our industrial partners will use this assay to screen 2 million chemically diversified small molecules to identify novel nonpeptidic inhibitors of HCV production.

PEPTIDE INHIBITORS OF CORE DIMERIZATION

We also designed an amplified luminescent proximity homogeneous assay to monitor core-core interactions on the basis of donor and acceptor beads that respond to the specific tags on the proteins. Using this assay, which has a high signal-to-background ratio, we screened 14 18-residue-long peptides derived from the HCV core and identified 2 partially overlapping peptides, SL-173 and SL-174, which caused 68% and 63% inhibition, respectively, of core106 dimerization.

SL-175, a 3-residue shorter version of SL-173, inhibited core106 dimerization by 50%, with a 50% inhibitory concentration of 22 μ M. Using fluorescence polarization, we found that a fluorophore-coupled deriva-

tive of SL-175 bound to core106 with a dissociation constant of 1.9 μ M and was displaced by the uncoupled peptide, with a 50% inhibitory concentration of 18.7 μ M. In a collaborative surface plasmon resonance study with J.-P. Lavergne, Centre National de la Recherche Scientifique, Lyon, France, SL-175 bound core169 with a dissociation constant of 7.2 μ M.

PEPTIDE INHIBITORS OF HCV RELEASE FROM HEPATOMA CELLS

When added to Huh-7.5 hepatoma cells infected with the HCV genotype 2a J6/JFH-1 or the 1b CG strain, peptides SL-173 and SL-175 prevented release of newly formed infectious HCV into the medium. Using a different approach, F.V. Chisari and his group, Department of Molecular and Experimental Medicine, independently found that SL-173 inhibits HCV focus formation *in vitro* by more than 90% and viral RNA synthesis 11-fold, 72 hours after infection. The combined results of our 2 groups thus establish that the HCV core is a useful novel target for development of anti-HCV drugs.

HEPATOMA CELL CULTURE SYSTEM FOR HCV GENOTYPE 1B

The culture system for HCV used routinely in laboratories worldwide was derived from strain JFH-1 of an HCV of genotype 2a. In Western countries, however, the most prevalent infections are caused by HCV strains of genotype 1. To understand differences between HCV of different genotypes and further evaluate potential inhibitors of protein-protein interactions in HCV or between HCV and human host proteins, we developed and characterized a unique culture system for the HCV genotype 1b CG strain. Two different protocols have been developed: the first one is based on the coculture of infected, virus-releasing cells with uninfected cells in a 2-chamber system; the second is a direct incubation of uninfected cells with supernatant from cells electroporated with the RNA from strain CG or from infected cells. Using either system, we have confirmed transfer of infectivity in several passages. Furthermore, we showed that cyclosporine A has comparable inhibitory effects on J6/JFH-1 and CG strains of HCV and that antibodies to CD81, a coreceptor for HCV, block infectious particles released into the supernatant of cells infected by the 1b CG strain. Initial results also suggest that the 2 strains are diversely affected by IFN- α ; J6/JFH-1 appears to be sensitive, and CG appears to be resistant. This finding, if confirmed, would reflect the situation in patients; patients infected with genotype 2 HCV generally respond better to interferon treatment than do patients infected with genotype 1 HCV.

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Hepatitis C Virus RNA Replication and Virion Assembly

T.L. Tellinghuisen, J.C. Treadaway, K.L. Foss, I. Angulo-Herrera

Hepatitis C virus (HCV) is a human pathogen of global importance; according to some estimates, nearly 3% of the world's population are chronically infected. Long-term viral replication in these individuals leads to severe liver disease, including cirrhosis and, often, hepatocellular carcinoma. The current treatment with agents nonspecific for HCV is poorly tolerated and is ineffective in about half of the patients, emphasizing the need for effective antiviral drugs specific for the virus.

The HCV replicase, the multicomponent machine that replicates the viral RNA, is an ideal drug target. The core replicase consists of 5 HCV proteins associated with well-characterized polymerase, protease, and helicase activities. Some HCV replicase proteins, such as NS5A, are essential for HCV replication; however, their specific functions remain enigmatic.

We have been characterizing NS5A. Our goal is to understand the role of this protein in replication and, more generally, the replicase itself. We have defined NS5A as an essential, 3-domain metalloprotein component of the replicase. Our crystal structure of domain I of NS5A has provided a glimpse of the potential interactions of NS5A in the viral replicase.

We recently identified all of the amino acids in the poorly understood domains II and III that are required for HCV RNA replication. Additionally, we have discovered an interaction between the membrane anchor of NS5A and the protein NS4B, another component of the replicase. This interaction appears to localize NS5A to the replicase and is essential for RNA replication. We are identifying regions of NS5A whose functions are required for the production of infectious virus but not for RNA replication. Our findings suggest that NS5A may function as a regulator of the switch between RNA replication and virus production. We are conducting biochemical, genetic, and structural experiments to evaluate the potential interaction surfaces and activities of NS5A observed in our previous structural and genetic

work. We have also begun applying high-throughput genetic screens to identify required host components of the replicase.

Our ultimate goal is to understand, at the molecular level, the assembly, activity, and regulation of the HCV RNA replication machinery. Greater insight into the poorly understood replicase components, such as NS5A, will provide a more complete view of the replicase complex and will fuel the design of new drugs.

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Strain and Species Barrier Phenomena in a Cell-Free System

J. Castilla, N. Fernández-Borges, J. de Castro

Transmissible spongiform encephalopathies are fatal neurodegenerative disorders that affect both humans and animals. The disorders can be classified as genetic, sporadic (putatively spontaneous), or infectious. The infectious agent associated with these encephalopathies, the prion, appears to consist of the single protein PrP^{Sc}, an abnormal conformer of the natural host protein PrP^C. Prions propagate by converting host PrP^C into PrP^{Sc}.

One characteristic of prions is their ability to infect some species and not others. This phenomenon is known as the transmission barrier. Interestingly, prions occur in the form of different strains with distinct biological and physicochemical properties, even though all the strains have the same PrP amino acid sequence, albeit in presumably different conformations. In general, the

transmission barrier is manifested as an incomplete attack rate (i.e., the percentage of animals in a group in which disease develops after inoculation with prions is less than 100) and long incubation times (time from inoculation to the onset of the clinical signs of disease), which become shorter after serial passages of the prion strain in animals. Compelling evidence indicates that the transmission barriers are closely related to differences in PrP amino acid sequences between the donor and recipients of the infectious prions and the prion strain conformation.

Unfortunately, the molecular basis of the transmission barrier and its relationship to prion-strain conformations are currently unknown, and we cannot predict the degree of a species barrier simply by comparing the prion proteins from 2 species. We have conducted a series of experiments in which we used protein misfolding cyclic amplification, a technique that mimics in vitro some of the fundamental steps involved in prion replication in vivo, albeit with accelerated kinetics. The in vitro generated prions have key prion features: they are infectious in vivo and maintain their strain specificity. We have used this technique to efficiently replicate a variety of prion strains from, among others, mice, hamsters, bank voles, deer, cattle, sheep, and humans. The correlation between in vivo data and our in vitro results suggests that protein misfolding cyclic amplification is a valuable tool for assessing the strength of the transmission barriers between diverse species and for different prion strains. We are using the method to determine which amino acids in the PrP^C sequence contribute to the strength of the transmission barrier.

These studies are useful in evaluating the potential risks to humans and animals not only of established prion strains but also of new (atypical) strains. For example, although the prion strain that causes classical sheep scrapie cannot cross the human transmission barrier in vitro, the strain that causes bovine spongiform encephalopathy can cross the human transmission barrier efficiently after propagation in sheep. In addition, we have generated prions that are infectious to species hitherto considered resistant to prion diseases.

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